Genetic Diversity Within and Among Populations of Black Robins on the Chatham Islands, New Zealand

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Abstract

Endemic island populations worldwide are at greater risk of extinction than similar mainland populations, in part due to the specific genetic threats faced by small populations, namely of loss of genetic diversity and inbreeding. Reduced genetic diversity limits the ability of populations to adapt to altered conditions, while unavoidable inbreeding reduces population fitness through the effects of inbreeding depression. Effective conservation management requires the understanding of these effects on populations of interest to adopt appropriate strategies to reduce such threats, and thereby ensure long-term population persistence.

Through the use of next-generation sequencing, I isolated 11 polymorphic microsatellite loci to allow analysis of current levels of genetic diversity in the endangered Chatham Island black robin *Petroica traversi*. The black robin has a history of small population size, including a population bottleneck of a single breeding pair, prior to recovery of population size over the past 30 years. The species is currently limited to populations on two small islands, and likely has a high extinction risk due to unavoidable inbreeding in the recovering populations, and may have experienced loss of genetic diversity due to strong genetic drift within these small populations. I compared levels of genetic diversity in the black robin to that of its closest congener, the Chatham Island tomtit *Petroica macrocephala chathamensis*, to assess how the population history of the black robin has affected its genetic diversity. Additionally, I compared levels of diversity between island populations of each species, to determine whether the smaller populations experienced lower diversity and therefore greater extinction vulnerability. Genetic diversity was lower in the black robin than the tomtit, and lower in the smaller populations of both species. The detection of levels of genetic diversity in the tomtit similar to those of threatened species suggests population viability of this species of least concern may be lower than expected. The two island populations of black robin are thought to have been isolated from one another for 26 years, and so populations were genotyped to determine whether this isolation has resulted in population differentiation, despite the short period of isolation. The two populations show substantial genetic differentiation, indicating genetic drift has had strong independent effects on these isolated populations. Although the tomtit exists on three islands, there was no

evidence of current dispersal between the two populations assessed, and there was a similar level of differentiation between these populations and the black robin populations.

Over 30 years of observational data show the black robin to be socially monogamous, with no evidence of extra-pair breeding. However, assessment of the social pedigree using microsatellite genotyping found a conservative rate of extra-pair paternity of approximately 14%, and the existence of a low-level of intraspecific brood paternity could not be rejected. As yet, the reason for the evolution of a strategy of extra-pair paternity is unknown.

From the results of this study, I recommend reciprocal translocations of black robins between island populations as a form of assisted gene flow to bolster genetic diversity of each population, and to reduce inbreeding in the smaller of the two populations. Furthermore, the establishment of a third population is recommended to minimise extinction vulnerability of this endangered species. As the black robin is not genetically monogamous, selection of individuals for translocation will require the use of molecular techniques to assess relatedness, rather than the social pedigree, to maximise success.

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The candidate (Natalie Forsdick) completed all primer testing, optimisation and genotyping, as well as all data analyses and writing of the chapter. This constituted at least 95% of the work reported in this chapter.

Co-author contributions: Stinus Lindgreen (bioinformatician) cleaned up, collapsed and converted the Illumina reads to FASTA format, Marie Hale designed primers from the collapsed reads and Melanie Massaro supplied samples for the work.

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General Introduction

1.1 Extinction risks in island populations

Extinction can be caused by a range of stochastic (demographic, genetic, environmental) or deterministic (habitat loss, overexploitation, predation by introduced species, pollution) factors (Caughley 1994; Frankham 2005; Lande 1993). Small populations endemic to islands are inherently at greater risk of extinction than similar populations on the mainland (Frankham 1998). Worldwide, of 128 bird species that have become extinct during the last 500 years, 122 were island species (Loehle & Eschenbach 2012). Island endemic populations are more vulnerable to processes that could rapidly eradicate the entire population due to their limited geographic range, such as the introduction of novel predators or diseases (Blackburn *et al.* 2004). Furthermore, a range of demographic or genetic factors can increase extinction risk of small populations, including reduced genetic variation, accumulation of deleterious alleles, imbalanced sex ratios, and the inability to adapt to environmental change through loss of genetic diversity (Frankham 1998, 2005; Lande 1988).

1.2 New Zealand small populations

The New Zealand avifauna provides an example of the strong correlation between the level of endemism and extinction (Duncan & Blackburn 2004), with 51 endemic bird species becoming extinct following human arrival around 700 years ago (Holdaway *et al.* 2001). Currently, 50% of endemic New Zealand bird species are estimated to be at risk of extinction (Craig *et al.* 2000), surviving in small populations on offshore islands, or in small forest fragments on the mainland. The high level of endemism and the unique characteristics of many New Zealand bird species mean that these extinctions have a large impact on total biodiversity. For example, the extinction of all moa species resulted in the loss of an entire order of birds (*Dinornithidae*) (Holdaway *et al.* 2001). The ways in which genetic factors contribute to species extinctions has often been overlooked in New Zealand conservation projects, with the main focus usually on the immediate threat of predation (Craig *et al.* 2000; Jamieson *et al.* 2006). As predators have been eradicated from some island and mainland

sanctuaries (e.g. Tiritiri Matangi Island, Karori Wildlife Sanctuary; Galbraith and Cooper (2013), Ruarus *et al.* (2011)), genetic factors may now receive greater attention.

1.3 Genetic drift and inbreeding

Two key genetic processes may compromise survival of small populations: loss of genetic diversity over time due to genetic drift, and increased inbreeding leading to inbreeding depression (Keller & Waller 2002; Nei *et al.* 1975; Wright *et al.* 2008). Genetic drift is the fluctuation in allele frequencies between generations due to stochasticity (Lande 1988). Alleles can drift to fixation (i.e., their frequency in the population is equal to one when all individuals are homozygous for that allele) or extinction (a frequency of zero), resulting in reduced genetic diversity (Lande 1988; Lynch *et al.* 1995). Usually the rarest alleles will be lost in each generation, but when populations are maintained at low size over long periods, random loss of alleles over many generations can also eliminate relatively common alleles, and greatly reduce total variation (Frankham 2005; Lande 1988). Allelic diversity is more susceptible to such chance processes than heterozygosity, as rare alleles have a high likelihood of being lost from the population in a short period of time, and rare alleles contribute little to the level of heterozygosity while all alleles contribute equally to allelic diversity (Allendorf 1986). As random fluctuations in allele frequencies are much greater in small populations (Allendorf *et al.* 2013), genetic drift has a much stronger effect than in large populations, potentially overwhelming natural selection (Lande 1988; Lynch *et al.* 1995). In large populations, natural selection effectively removes mutations with large deleterious effects, and prevents those with small deleterious effects from becoming common (Lande 1994; Lynch *et al.* 1995). While beneficial, neutral, and harmful alleles are lost at relatively equal rates through genetic drift, their random nature means mutations are more commonly detrimental than neutral or beneficial, and so there is a higher likelihood of deleterious alleles becoming fixed through drift (Frankham 2005; Lynch & Gabriel 1990). This may contribute to an increase of the genetic load of the population. Genetic load describes the accumulation of deleterious recessive alleles in the population, and results in a reduction of the average fitness of the population as compared to the maximum fitness possible (Hedrick *et al.* 2014; Kirkpatrick & Jarne 2000; Lynch *et al.* 1995).

This reduction in fitness due to increased genetic load may reduce population growth, resulting in an ever smaller population experiencing ever stronger drift, a process known as mutational meltdown (Lynch *et al.* 1995; Lynch & Gabriel 1990), which can drive populations towards extinction. While drift has a strong effect in small populations, this process still takes time to occur, and extinction due to stochastic or demographic processes may occur before this process is recognised (Gilligan *et al.* 1997).

The actual population size may not accurately represent the risk of a population going extinct via genetic drift. Skewed sex ratios and breeding success may reduce the effective population size, and this may exacerbate the effects of drift (Jamieson 2011; Miller *et al.* 2009). The effective population size (N_e) is the size of an ideal population that loses heterozygosity through drift and inbreeding at the same rate as the true population (N), and can be used to predict the effects of inbreeding and drift in wild populations (Frankham 1995; Wright 1938). N_e is typically much smaller than N, with an estimated average ratio of N_e to N of 0.1, due to population fluctuations, variance in family size, and skewed sex ratios resulting in a limited number of individuals contributing alleles to the next generation (Frankham 1995; Lynch *et al.* 1995; Wright 1938).

Aside from long-term small population size, substantial reductions in genetic variation can also occur during short-term population bottlenecks (Lynch *et al.* 1995). During the founding of a new population, a small number of individuals carry a random subset of the genetic variation from the source population (Frankham 1997). The initial founding event may not be the only bottleneck the population experiences. Stochastic or deterministic events (including storm events, random population fluctuations, or skewed sex ratios) may reduce population size multiple times, and it may take many generations for the genetic health of the population to recover, particularly if the growth rate is low (Nei *et al.* 1975). For populations that have existed at small size over long periods of time, extreme bottlenecks may have a lesser effect on genetic diversity than the long period of small size (Nei *et al.* 1975). While allelic diversity is particularly vulnerable to population fluctuations, heterozygosity is more resilient and may not be substantially reduced during short-term bottlenecks (Allendorf 1986). Even when population size is reduced to two individuals for one generation, 75% of the total

heterozygosity will be maintained (on average) in the recovering population (Allendorf 1986).

While genetic diversity can be eroded by genetic drift, the only mechanisms that can increase genetic variation in island populations are immigration and mutation (Frankham 1997). Immigration may be limited for isolated populations, such as island populations, preventing the contribution of novel variation (Frankham 1997; Slatkin 1897). This may result in significant differentiation between populations over time, as drift acts independently on isolated populations (Slatkin 1987). The occurrence of novel mutations can act to increase variation in populations (Nei *et al.* 1975). As the process of mutation occurs slowly, the loss of variation through drift may drive small populations to extinction before mutation can replace it (Bijlsma *et al.* 2000; Frankham 2005; Nei *et al.* 1975). Although new mutations may increase diversity, they may also reduce fitness (Lynch & Gabriel 1990). However, such detrimental mutations may become advantageous under altered conditions, and so are an important component of adaptive potential (Allendorf *et al.* 2013).

In addition to genetic drift, inbreeding (i.e., mating amongst relatives) may increase the extinction risk of small populations (Keller & Waller 2002). Inbreeding should be a rare occurrence due to inbreeding avoidance mechanisms present in many species, such as kin recognition or dispersal from the natal site (Pusey & Wolf 1996). However, limited mate choice in small populations may result in inbreeding becoming unavoidable (Keller & Waller 2002). Close relatives share more alleles than randomly mating individuals, and so offspring produced via inbreeding will have more alleles identical by descent (i.e., homozygous) than those resulting from random matings (Charlesworth & Charlesworth 1999; Hedrick & Kalinowski 2000). While inbreeding increases homozygosity in the population, in contrast to drift it has no direct effect on allele frequencies (Charlesworth 2003). The increase in homozygosity caused by inbreeding results in reduced fitness in inbred populations, known as inbreeding depression (Charlesworth & Charlesworth 1987; Crnokrak & Roff 1999). There are two mechanisms by which this may occur: firstly, increased homozygosity results in increased expression of recessive deleterious alleles (Lynch *et al.* 1995; Ralls *et al.* 1988), and secondly, increased homozygosity reduces the expression of overdominance, where there is a heterozygote advantage (Charlesworth & Charlesworth 1999). As inbreeding becomes

more frequent in small populations, population fitness declines, resulting in lower rates of reproduction and survival (Keller & Waller 2002). Population size will decrease in each successive generation resulting in a positive feedback loop, whereby the reduction in population size will increase the frequency of inbreeding, further reducing population size, until the population becomes extinct (Jiménez *et al.* 1994; Keller 1998; Saccheri *et al.* 1998). In birds, inbreeding depression can lead to declines in sperm quality, fecundity, breeding success, and survival (Brekke *et al.* 2010; Grueber *et al.* 2010; Hemmings *et al.* 2012; Jamieson *et al.* 2003; Mackintosh & Briskie 2005). For example, a meta-analysis including 51 threatened bird species found that the severity of population bottlenecks was related to increased hatching failure rates (Heber & Briskie 2010).

Although inbreeding may have negative effects on population fitness, long-term inbreeding is hypothesised to reduce the effects of inbreeding depression (this process is known as purging), as natural selection should remove the expressed deleterious mutations from the gene pool (Bijlsma *et al.* 2000; Miller & Hedrick 2001; Saccheri *et al.* 1996). However, while experimental studies may be able to create conditions necessary for effective purging (Miller & Hedrick 2001), there is little evidence of its effectiveness in wild populations (Keller & Waller 2002; Kennedy *et al.* 2014; Leberg & Firmin 2008). Inducing purging by encouraging intentional inbreeding in populations of conservation interest is not recommended, as the increased short-term inbreeding depression is more likely to reduce population viability (Edmands 2007; Jamieson *et al.* 2006; Leberg & Firmin 2008).

The reduction in genetic variation resulting from genetic drift and inbreeding has negative consequences for the long-term survival of species, and limits the ability of populations to adapt to changing environmental conditions (Allendorf *et al.* 2013; Frankham 2005). Populations with low diversity may rapidly be wiped out by novel diseases or parasites, as they may not possess any genotype for resistance (McCallum 2008). The importance of adaptive potential is widely recognised in the face of climate change, due to temperature changes and rising sea levels altering habitat ranges, migratory routes, and feeding patterns (Chevin *et al.* 2010; Reed *et al.* 2011). An example whereby a population has experienced a severe bottleneck and consequently has reduced adaptive potential, is that of the Mauritius kestrel (*Falco punctatus*). The population of the Mauritius kestrel fell to a single breeding

pair in 1974, but recovered to over 400 individuals by 1997 following conservation management (Groombridge *et al.* 2000). The population displayed reduced diversity as compared to museum samples and related kestrel species (Groombridge *et al.* 2000). While the population avoided extinction, it may be more prone to extinction in the future due to the introduction of novel disease and lack of evolutionary potential preventing adaptation.

1.4 Translocations

Translocation is a conservation management tool that can be used to mitigate the effects of genetic drift and inbreeding depression. Translocations are commonly used in New Zealand conservation projects, particularly to remove the pressure of predation (Miskelly & Powlesland 2013). Translocations involving 41 species of birds have resulted in the successful establishment of new populations, and five species now exist only as translocated populations (Chatham Island black robin *Petroica traversi*, buff weka *Gallirallus australis hectori*, kakapo *Strigops habroptilus*, little spotted kiwi *Apteryx owenii*, and the South Island saddleback *Philesturnus carunculatus*) (Miskelly & Powlesland 2013). Modelling may assist in determining appropriate numbers and frequencies of translocation events to minimise risks to existing populations, yet capture sufficient genetic variation to ensure the long-term viability of translocated populations (e.g., Lacy (2000); Weiser *et al.* (2012)).

Translocations also can be used to assist gene flow between otherwise isolated populations. Population fitness and persistence can be improved through the introduction of unrelated individuals, resulting in reductions in the genetic load and the frequency of inbreeding (Hedrick *et al.* 2014; Ingvarsson 2001; Vilà *et al.* 2003; Vucetich *et al.* 2005; Weeks *et al.* 2011). Individuals produced through outbreeding should experience improved fitness, known as heterosis, due to a reduction in homozygosity and the associated reduced expression of recessive deleterious alleles (Ingvarsson 2001). A key example of the success of such translocations can be seen in the Florida panther (*Puma concolor coryi*) (Pimm *et al.* 2006). Populations in Florida had declined throughout the 1900s, and the remaining population exhibited reduced genetic diversity and a variety of potentially deleterious traits, including cryptochordism, kinked tails, and extremely poor sperm quality (Pimm *et al.* 2006). Eight females from the most closely related population in Texas were introduced to the Florida panther population (Pimm *et al.* 2006). Following this translocation of females, the frequency

of these negative traits decreased, and the population grew substantially (Pimm *et al.* 2006). This demonstrated that translocation of unrelated individuals could mask the negative impacts of the genetic load, and improve population viability. In New Zealand, reciprocal translocations of South Island robins between island populations resulted in improvements in fitness-related traits (Heber & Briskie 2013). Translocations conducted in 1973 had established two populations from very small numbers of founders on Motuara and Allports islands, and these populations experienced hatching failure and reduced immunocompetence compared to mainland populations (Heber & Briskie 2013). Reciprocal translocations of 25 females were carried out between the two island populations, resulting in improvements in juvenile survival, pairing success, and immunocompetence, and lower levels of sperm abnormalities (Heber & Briskie 2013).

1.5 Genetic data

Genetic data is used to answer questions involving population genetic processes, including those relating to genetic diversity and genetic structuring. Microsatellites are one of a number of genetic markers that can be used. Microsatellites are short tandem base-pair repeats (also known as simple sequence repeats (SSRs) or short tandem repeats (STRs)) found at high frequency throughout the nuclear genome (Balloux & Lugon-Moulin 2002; Selkoe & Toonen 2006). These repeating sequences can be very polymorphic, as they have high mutation rates due to enzyme slippage during replication, which alters the number of repeats in the sequence (Selkoe & Toonen 2006). The differences in lengths of these microsatellite sequences can be measured through amplification and genotyping using microsatellite primers. Primers are short sequences developed to bind to the highly-conserved flanking regions on either side of the microsatellite sequence. As these flanking regions are highly-conserved within and between related species, identical primer sequences may be used for genotyping in several related species, allowing cross-species comparisons (Engel *et al.* 1996). However, the ascertainment bias, where lower levels of polymorphism are expected in non-target species due to the preferential selection of highly polymorphic loci in the target species, may limit the use of these primers in more distantly related species (Selkoe & Toonen 2006). The high levels of diversity within microsatellite regions, and their codominance, means that a small number of loci can provide sufficient information to answer many population genetic questions posed. Therefore, microsatellites are a relatively cost-effective marker that can be

used for many different analyses, from determining levels of genetic diversity within populations, genetic structuring, rates of migration, analysis of paternity, and individual identification (Guichoux *et al.* 2011).

Microsatellites are frequently used in population genetic studies as their rapid rate of mutation results in high levels of variation. The mutation rate of microsatellites has been estimated at between 10^{-2} and 10^{-6} mutations per locus per generation, and is considerably higher than genomic markers such as SNPs (single nucleotide polymorphisms, 10^{-9} mutations per locus per generation) (Guichoux *et al.* 2011; Selkoe & Toonen 2006). Consequently SNPs have lower allelic diversity, requiring the use of a much larger number of loci to obtain the same amount of information (Guichoux *et al.* 2011). However, the high mutation rate of microsatellites may result in spontaneous mutations that may complicate pedigree analysis (Guichoux *et al.* 2011), and may prevent detection of historical population events (Selkoe & Toonen 2006). Furthermore, microsatellites have a complex mutational process, and methods of analysis may not accurately estimate this (Selkoe & Toonen 2006).

New techniques of next-generation genomic sequencing to identify microsatellite sequences allow a greater number of microsatellites to be detected at lower cost and in a shorter timeframe than previous methods (Abdelkrim *et al.* 2009; Castoe *et al.* 2012; Guichoux *et al.* 2011). With a greater number of sequences detected, larger microsatellites (tri-, tetra-, and pentanucleotide repeats) can be preferentially selected for primer design, as these are less prone to enzyme slippage during amplification than dinucleotide repeats, which often result in 'stutter' bands (Guichoux *et al.* 2011). While new genomic methods are increasing in popularity, analyses based on small numbers of polymorphic microsatellite loci remain sufficient to answer many questions related to population genetics, and so microsatellites remain the most popular marker (Guichoux *et al.* 2011).

1.6 Extra-pair paternity

Highly variable genetic data (such as microsatellite genotypes) can be used to infer parentage of individuals and to create pedigrees (Jones *et al.* 2010). Pedigree information is useful in conservation projects to record breeding success, investigate breeding systems, and also to

infer relatedness between individuals, allowing estimation of inbreeding depression, and enabling management decisions that minimise inbreeding (Pemberton 2008). Behavioural data collected from observations can be used to create pedigrees, but may not always be accurate, due to limited access to breeding areas, difficulties with individual identification, and the often short duration of mating episodes. Genetic data may be beneficial to ensure the accuracy of observational data and so may improve the accuracy of the pedigree (Pemberton 2008). A range of methods are available to perform parentage analysis, including methods of exclusion, allocation, probability analysis, and reconstruction (Jones *et al.* 2010).

When genotypic data does not match observed pedigrees, it may indicate extra-pair parentage. Extra-pair parentage occurs when one member of the social pair seeks copulations outside of the social pair, resulting in successful fertilisation (Møller 1986). Social monogamy is common in birds, yet true monogamy is rare, with 90% of bird species estimated to produce extra-pair offspring (Griffith *et al.* 2002). Extra-pair paternity (EPP) is particularly common, with rates of up to 55% in some bird species (Griffith *et al.* 2002). There are a number of hypotheses for the evolution of EPP as a mating strategy by females (Arct *et al.* 2015), including gaining direct benefits (resources) for offspring, limiting effects of infertility in the social mate (Wetton & Parkin 1991), improving genetic diversity of offspring (Westneat *et al.* 1990), avoiding the negative fitness effects of inbreeding (Foerster *et al.* 2003; Reid *et al.* 2015), maximising genetic compatibility with the father (Kempenaers *et al.* 1999; Tregenza & Wedell 2000), or obtaining beneficial genes for the offspring (Møller 1988; Westneat *et al.* 1990). EPP may not be detected through observation, and this may contribute to errors in pedigrees created from observational studies, resulting in inaccurate estimates of relatedness, and in analyses based on relatedness (Pemberton 2008; Reid *et al.* 2014). Therefore, molecular methods may be used to assess parentage to ensure effective conservation decision-making involving breeding or translocations.

1.7 History of the Chatham Island black robin

One of New Zealand's many endemic bird species is the Chatham Island black robin (*Petroica traversi*) (Order: Passeriformes, Family: Petroicidae), a small (approx. 20 g) insectivorous passerine endemic to the Chatham Islands, 800 km east of New Zealand (Massaro *et al.* 2013a; Miller & Lambert 2006). Prior to human arrival around 500 years ago,

the black robin was present on five islands in the Chatham's archipelago: Chatham, Pitt, Rangatira (South East/Hokorereora), Mangere, and Little Mangere islands (Butler & Merton 1992; Kennedy 2009).

Following human arrival, predation by exotic predators (particularly by rodents and cats), and landscape modification for farming led to reductions in numbers (Kennedy 2009). By the 1890s, the species is thought to have been extirpated from four islands, and was restricted to Little Mangere Island, and remained this way until the late 1970s (Butler & Merton 1992; Kennedy 2009). Kennedy (2009) estimates that during this period, Little Mangere Island could support a population of no more than 35 birds. Population surveys of birds on the Chatham Islands commenced in the late 1960s and recognised the vulnerability of the black robin population (Butler & Merton 1992). In the first census in 1968, the black robin population was estimated to number thirty birds, although this is thought to have been an over-estimate, due to counting difficulties that continued until all robins were colour-banded in 1975 (Butler & Merton 1992; Kennedy 2009). The first full census of all colour-banded robins was conducted in 1976, and found only seven robins remaining on Little Mangere (Kennedy 2009). The black robin population was in a critical state, and conservation action was required.

The seven remaining robins were translocated to the larger and more accessible Mangere Island in 1977 (Butler & Merton 1992). However, low reproductive output and offspring survival resulted in only five juveniles surviving over the next three years. Furthermore, food stress during the summer resulted in adult mortality, and the population fell below the replacement rate (Butler & Merton 1992; Kennedy 2009). In 1980, the Chatham Island robin became known as the world's most endangered bird, with only five individuals remaining, including a single successfully breeding pair (Butler & Merton 1992). Over the next decade, a programme of supplementary feeding, parasite control, and nest box provision was established (Butler & Merton 1992). Ultimately the practice of cross-fostering eggs into nests of the Chatham Island tomtit (*Petroica macrocephala chathamensis*) saved the species (Butler & Merton 1992). By removing newly-laid eggs from black robin nests, the birds were encouraged to lay additional clutches, resulting in more offspring being raised to fledging than would be naturally possible (Butler & Merton 1992).

As the population began to recover, the management team started to consider establishing a second population (Butler & Merton 1992). There were three main reasons to establish a second population. Firstly, Robin Bush on Mangere Island would eventually provide insufficient habitat and would limit short-term population growth. Secondly, better management of breeding pairs was required. The team recognised that there was little they could do to minimise inbreeding in this already unavoidably inbred population. Separating inbred pairs could best be managed if individuals could be removed to a separate island, and a second breeding line could be established. Lastly, a second population could act as insurance against catastrophic events. Severe storms or the introduction of disease could easily wipe out a single small population, but may be less likely to affect both island populations.

Rangatira Island was selected for the establishment of the second population as it was the site of the cross-fostering by the tomtits (Butler & Merton 1992). Between 1983 and 1990, approximately 76 birds were translocated to Rangatira Island (Kennedy 2009). No further translocations (or cross-fostering) were carried out after the intensive management programme ended in 1990 (Butler & Merton 1992; Kennedy 2009). Black robin numbers have continued to increase following the end of active management (Kennedy 2009), demonstrating that the species can survive on its own. The rescue of the Chatham Island black robin from seemingly-inevitable extinction is touted as a conservation success (Butler & Merton 1992; Miskelly & Powlesland 2013).

1.8 Current state of the black robin populations

Currently, the black robin numbers approximately 290 individuals on two islands, with 200 – 250 individuals on Rangatira Island, and around fifty on Mangere Island (see Figure 1.1) (M. Massaro, personal communication), and is classed as Endangered (BirdLife International 2016b). The population on Mangere Island appears to be at carrying capacity, due to slow forest regeneration preventing further growth (Kennedy 2009). The robin can breed in its first year, laying one clutch of one to three eggs, and can live to at least 14 years (Butler & Merton 1992). Regular storm events and strong winds coupled with an aversion to flying across open

areas prevent the robin from flying the 11 km distance between the two islands it inhabits, with no evidence of natural dispersal since the Rangatira population was established in the 1980s (Kennedy 2009).

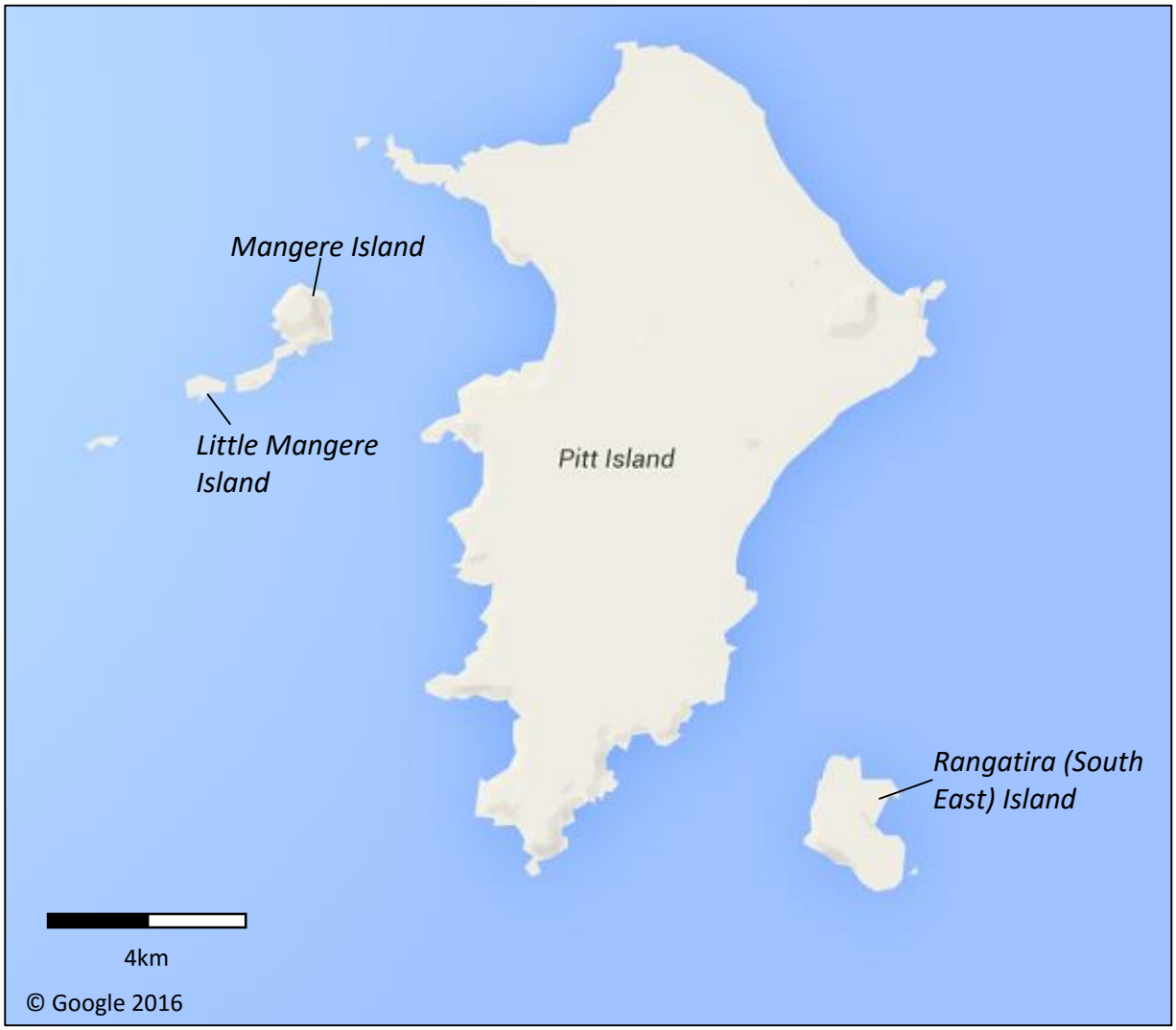

Figure 1.1 Map of the southern islands in the Chatham's archipelago. Pitt Island is the second largest island within the archipelago, with around 40 human inhabitants. The Chatham Island black robin is currently restricted to Mangere and Rangatira islands.

Although the recovery of the black robin population demonstrated that even species on the brink of extinction can be saved, in the first ten years following the bottleneck severe inbreeding took place with frequent brother-sister and father-daughter matings (Massaro *et al.* 2013a). These high rates of inbreeding had impacts on population fitness, whereby juvenile

survival is reduced and there is no evidence of purging (Kennedy *et al.* 2014; Weiser *et al.* 2016). A previous study of black robin genetics found the black robin exhibited low minisatellite diversity (Ardern & Lambert 1997). Minisatellites are tandem motifs of 6 – 100 base pairs with repeats of varying lengths (Vergnaud & Denoeud 2000). The lower error rate in replication of these large motifs results in slower mutation of minisatellites compared to microsatellites, and so minisatellites are less polymorphic and less informative than microsatellites (Vergnaud & Denoeud 2000). The longer sequence lengths also require higher quality DNA than microsatellites, and as minisatellite polymorphisms are detected using gel electrophoresis, only a limited number of samples can be compared accurately (Vergnaud & Denoeud 2000). Use of more highly informative microsatellite markers would allow more accurate quantification of diversity, and also detection of fine-scale population structure.

Current management goals include the establishment of a third population of black robin, potentially on Pitt or Little Mangere islands, to minimise the impact of environmental stochasticity (Department of Conservation [DOC], 2001a). Reciprocal translocations of birds between the islands may also be beneficial to maintain genetic diversity in the existing populations, if the two island populations are genetically differentiated. Conducting population viability analyses would be useful to determine the number of individuals required to establish a self-sustaining population on a third island, such as Pitt Island. One such study using the AlleleRetain model (Weiser *et al.* 2012) recommended that establishment of such a population on Pitt Island would require at least forty individuals to ensure persistence of a self-sustaining population and the retention of at least 90% of allelic diversity over the next 100 years (Weiser 2014). Additional simulations suggested translocation of one individual between the Rangatira and Mangere populations every two to ten years to maintain at least 90% of the unique alleles in both populations over the next 100 years (Weiser 2014). However the accuracy of this modelling may be limited, as it is based on hypothesised levels of allelic diversity derived from simulations of allelic diversity at the time of the bottleneck in 1980. Two simulations were presented; one with relatively high founder diversity following the bottleneck (four founder alleles per locus), and a second with relatively low diversity (1.64 founder alleles per locus; Weiser (2014)). More accurate estimates of current diversity within the populations using microsatellites will improve this modelling, providing more accurate estimates of the number of individuals required for such translocations. Currently, there are only three polymorphic microsatellite loci known to amplify in the black robin, and

analysis using these loci in a previous study found levels of polymorphism too low to answer questions related to genetic diversity and differentiation (Cubrinovska *et al.* 2016).

1.9 The aims of this study

In this study I aim to use next-generation sequencing methods to develop microsatellite primers specific to the Chatham Island black robin to investigate the following:

1. To determine whether the likely isolated island populations have genetically differentiated from one another due to genetic drift acting on each population independently, and to clarify whether there is any migration between island populations.
2. To determine whether there are two populations of black robins on Rangatira Island, residing in distinct forest habitats.
3. To quantify the levels of genetic variation in terms of allelic diversity and heterozygosity in each population of black robins and to compare these with the black robin's sister-species, the Chatham Island tomtit to investigate how the different demographic histories have affected levels of diversity in each species.
4. To clarify whether the black robin is genetically monogamous, and determine the implications of this for the known pedigree, and future management practices.

In Chapter Two, I develop species-specific polymorphic microsatellite markers through the use of next-generation sequencing methods for the black robin, and test these for cross-species utility in the Chatham Island tomtit. The tomtit is the sister-species of the Chatham Island black robin, and the two coexist on Rangatira and Mangere islands (DOC, 2001b). Although the two species are similar, the current tomtit population is much larger than the black robin (around 700 – 900 individuals (DOC, 2001b)).

In Chapter Three, I use the markers described in Chapter Two to assess the levels of genetic variation within and genetic differentiation between island populations of the black robin. While Mangere Island has a much smaller land area and current population size, it is the origin of the more recently founded Rangatira population (Butler & Merton 1992). Genetic

drift will likely have had a strong impact on both of these small island populations, which may have resulted in population differentiation. The very small Mangere population is likely to experience stronger genetic drift than the population on Rangatira, and therefore may be continuing to lose genetic diversity at a faster rate. If there is substantial differentiation between these two populations, reciprocal translocations between islands may be required to bolster the level of genetic diversity within each population. Furthermore, if these island populations have differentiated, the potential establishment of a third population may require the input of individuals from both islands to capture the maximum range of diversity, and consequently improve viability of the new population. I then compare the level of genetic variation between the black robin and the tomtit, which has not experienced such a severe population bottleneck or period of inbreeding. I will also investigate whether there is population substructuring within the larger Rangatira black robin population. Several studies identify two distinct populations on Rangatira Island, one in the northern Woolshed Bush, and a second in the more southern Top Bush, and present separate analyses for each (Kennedy *et al.* 2014; Weiser *et al.* 2016). However, dispersal between these patches has been recorded (M. Massaro, unpublished data), and may present sufficient gene flow to prevent genetic differentiation. I will determine whether there are two genetically distinct populations on Rangatira Island, and as such, whether sourcing of individuals from both bush areas is required to capture the maximum diversity if a third population is to be established.

In Chapter Four, I assess the likelihood of extra-pair paternity in the black robin. If any translocations are to occur, appropriate selection of individuals is required to minimise inbreeding and maximise genetic diversity. If the actual relatedness of individuals selected for translocation differs from that assumed based on observational pedigree data, the success of both reciprocal translocations between populations and translocations to establish a new population may be compromised.

In Chapter Five, I make recommendations for reciprocal translocations of black robins between island populations, and the establishment of a third population to be addressed with renewed priority to ensure continued viability of this endangered endemic bird.

Chapter Two

Isolation and characterisation of microsatellite loci for the Chatham Island black robin *Petroica traversi*

Material presented in this chapter has been accepted for publication in Conservation Genetics Resources (March 2016). The accepted manuscript is reproduced in full in Appendix 1.

2.1 Abstract

The Chatham Island black robin *Petroica traversi* is an endangered species found only on Rangatira and Mangere islands. After declining to a single breeding pair in 1980, the recovery to a current species size of 290 individuals is regarded as a conservation success. However, the severity of the bottleneck may have had long-term consequences for the species due to the associated loss of genetic diversity and high level of inbreeding in the recovering population. In this study, microsatellite primers were developed using next generation (Illumina) sequencing, and were tested for cross-amplification in the closely related Chatham Island tomtit *Petroica macrocephala chathamensis*. The primers isolated here will be used to assess the level of genetic diversity within this species, and to measure differentiation between island populations.

2.2 Introduction

The Chatham Island black robin *Petroica traversi* is a small insectivorous passerine endemic to the Chatham Islands archipelago, 800 km east of New Zealand (Butler & Merton 1992). The black robin was historically found on five islands in the archipelago: Chatham, Rangatira, Pitt, Mangere, and Little Mangere islands. Following human arrival 450 – 500 years ago, habitat degradation and the introduction of exotic predators led to the decline of the species, and its eventual extirpation on all but the smallest island, Little Mangere, is estimated to have occurred by the late 1800s (Butler & Merton 1992; Kennedy 2009). A maximum of 35 individuals are estimated to have been supported on this 15 ha island

(Kennedy 2009). Population surveys carried out in 1971-72 recognised the vulnerability of this population, and conservation efforts began. However, the population continued to decline. By 1977, only seven individuals remained, and emergency action was required. All individuals were translocated to the larger and more accessible Mangere Island, where habitat restoration had begun. By the end of 1979, the entire species numbered five individuals, containing only a single successfully breeding pair, from which all individuals today are descended (Butler & Merton 1992). Intensive management over the next decade involved cross-fostering of eggs, provision of nest boxes, supplementary feeding, and the eventual translocation of individuals to the larger Rangatira Island, and prevented the extinction of the species. Today the species numbers approximately 290 individuals on the two islands (Melanie Massaro, personal communication). However, the long-term low population size and severe bottleneck has led to one of the highest rates of inbreeding in any known wild bird species (Ardern & Lambert 1997). Due to the small population size, high level of inbreeding, and limited habitat, the current IUCN Red List categorises the Chatham Island black robin as Endangered (BirdLife International 2016).

The black robin is averse to flying across open areas, and so the populations on Mangere and Rangatira are assumed to have been isolated from one another for 26 years. As the entire species is descended from a single pair, genetic variation within the species is expected to be low. However, the strong independent effects of genetic drift may have resulted in genetic differentiation between the two island populations. Understanding the current population genetic structure is essential to inform future management for this species, particularly to determine whether translocations of individuals between the two island populations would bolster levels of genetic diversity on either or both islands.

A recent study investigating potential hybridisation between the black robin and the Chatham Island tomtit *Petroica macrocephala chathamensis* using microsatellite markers found little genetic variation in the black robin (Cubrinovska *et al.* 2016). However, this previous study used markers designed for a different bird species. When microsatellite primers developed for non-target species are used, less diversity is typically found than in the target species due to ascertainment bias (the preferential selection of loci polymorphic in the target genome during primer development), and so these results may not be representative of the true variation

present (Galbusera *et al.* 2000; Primmer *et al.* 1996). While the loci used by Cubrinovska et al. (2015) were able to show the lack of recent hybridisation between the two species, the very low levels of genetic variation within and among black robin populations were insufficient to resolve questions relating to population structure.

Here I describe the development of primers for polymorphic microsatellite loci isolated from the black robin (*Petroica traversi*) genome, and their cross-amplification in the Chatham Island tomtit (*Petroica macrocephala chathamensis*). These new microsatellite loci will allow greater levels of variation to be detected than in previous studies, enabling the determination of fine-scale population structure in black robins and their sister-species, the Chatham Island tomtit.

2.3 Methods

Black robin DNA was extracted from blood spots of three individuals using a PureLink™ Genomic DNA Mini Kit (Invitrogen). DNA quantity was low, so DNA extracted from three individuals was pooled to ensure sufficient quantity (>2.5 µg) for next generation (Illumina) sequencing, conducted by New Zealand Genomics Ltd (NZGL). A MiSeq 250 bp paired-end run, with 550bp insert size, produced 9,596,842 raw read pairs. PAL_finder (v. 0.02.04) (Castoe *et al.* 2012) was used to search for reads containing potential microsatellites. PAL_finder was run with default settings except for the primer minimum temperature (PRIMER_MIN_TM=55) and the minimum number of n-mer repeats detected: (2mer - minimum 10 repeats; 3mer - minimum 8 repeats; 4mer - minimum 7 repeats). Reads containing microsatellites with sufficient overlap (at least 11 nucleotides) were then collapsed using AdapterRemoval 2.0 (Lindgreen 2012), and collapsed sequences containing microsatellites were used for primer design. Primer3 was then used to design primers for these microsatellite regions (Koressaar & Remm 2007; Untergasser *et al.* 2012). A variety of different repeat motifs were selected to test for amplification and polymorphism, in order to minimise the chance of selecting the same microsatellite from independent reads. Microsatellites were also selected based on the number of repeats, with the aim of selecting repeat numbers high enough to maximise the probability of polymorphism, while not so high that stutter would be likely to make scoring difficult (particularly for dinucleotide repeats). Primers for forty microsatellite regions (GenBank accession numbers KU194428-KU194467)

were thus selected for further testing: 11 containing a tetra-nucleotide, 13 containing a tri-nucleotide, and 11 containing a di-nucleotide. There were also five compound microsatellites. M13 tags (5'-TGTAAAACGACGGCCAGT) were added to the 5' end of forward primers for universal fluorescent dye labelling (Schuelke 2000). PIGtails (GTTTCTT) were attached to the 5' end of reverse primers to reduce adenylation (Brownstein *et al.* 1996).

DNA was extracted from blood spots or feathers of 30 black robins and 30 tomtits using an Invitrogen PureLink™ Genomic DNA Mini Kit. DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer following extraction. Concentrations ranged from 13.7 – 463.5 ng/µl for black robin samples, and 7.4 – 346.8 ng/µl for tomtits. Initial testing for amplification was carried out in 15 µl reactions containing 0.5 µl of genomic DNA, 1 x NH_4 reaction buffer (Bioline), 2 mM $MgCl_2$, 0.08 mM dNTPs, 0.33 µM of forward primer, 0.33 µM reverse primer, and 0.6 U BIOTAQ DNA polymerase (Bioline). An Eppendorf® Mastercycler® was used for PCR thermocycling. One of two thermocycle protocols were used: either a standard three-step protocol, or a touchdown (TD) protocol. The standard three-step protocol consisted of: 95°C for 12 min, 10 cycles of 94°C for 15 s, annealing temperature (T_A)° C for 30 s (see Table 2.1), 72°C for 30 s, followed by 30 cycles of 89°C for 15 s, T_A°C for 30 s, 72°C for 30 s, then a final extension of 72°C for 10 min. PCR products were visualised by electrophoresis on a 1.4% agarose gel pre-stained with SYBR® Safe. Primers were initially tested for optimal amplification at an annealing temperature calculated based on their melting temperatures, but these initial temperatures proved to be too low. Subsequently all primers were tested at T_A = 52°C, and T_A was adjusted as required to produce clear single bands (see Table 2.1). Loci that amplified inconsistently at a single annealing temperatures were then tested using a touchdown protocol, in an effort to include as many primers for genotyping as possible. The touchdown protocol consisted of: 95°C for 15 min, 10 cycles of 94°C for 15 s, (from 62°C to 53°C, decreasing 1°C per cycle) for 30 s, and 72°C for 1 min, then 25 cycles of 94°C for 15 s, 52°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 15 min.

Fluorescently labelled M13 primers (5'-TGTAAAACGACGGCCAGT, labelled with 6-FAM, NED, VIC, or PET, Applied Biosystems) were then added to PCR reactions for loci that amplified successfully, and loci were re-amplified for genotyping on an automated DNA

sequencer to assess variability. With M13 primers, 15 µl reactions contained 0.5 µl of genomic DNA, 1 x NH4 reaction buffer (Bioline), 2 mM $MgCl_2$, 0.08 mM dNTPs, 0.083 µM of forward primer, 0.33 µM reverse primer and 0.33 µM M13 primer, and 0.6 U BIOTAQ DNA polymerase (Bioline). When M13 primers were added, if initial $T_A \geq 54°C$, the protocol was altered to 95°C for 12 min, 10 cycles of 94°C for 15 s, T_A°C for 30 s, 72°C for 30 s, followed by 30 cycles of 89°C for 15 s, 53°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 10 min. This improved annealing of the M13 primer (Schuelke 2000), except when using PT2, PT24, and PT27, where the original protocol was used. $MgCl_2$ was increased to 2.33 mM to further improve annealing of PT2 and PT27.

PT24 and PT27 continued to amplify inconsistently, and so new forward and reverse primers were designed for these loci. New primers were tested for amplification in combination with the previous primer sets. For PT24, the new forward and reverse primers amplified most consistently, while in PT27 a combination of the original forward and new reverse primers, in combination with increased $MgCl_2$ of 2.33 mM, was most consistent. Primer sequences stated in Table 2.1 are the final combinations used.

Samples were prepared for genotyping by adding 0.5 µl PCR product to 0.3 µl Genescan 500LIZ size standard and 12 µl HiDi formamide. When loci produced only a faint band on the gel, PCR product was increased to 1.0 µl to ensure clear scoring of alleles. These were denatured at 95°C for five minutes. Genotyping was performed on an ABI Prism® 3130xl Genetic Analyser (Applied Biosystems). Allele sizes were scored visually using GeneMarker (v.2.20; SoftGenetics). At least six individuals of each species were genotyped for each locus to test for polymorphism. A total of thirty individuals of each species collected from Rangatira Island were amplified and genotyped for all loci that were polymorphic.

Testing for null alleles was carried out using MICROCHECKER ver. 2.2.3 (Van Oosterhout *et al.* 2004). Tests for deviation from Hardy-Weinberg equilibrium (HWE) and significant linkage disequilibrium (LD) were performed using ARLEQUIN ver. 3.5 (Excoffier & Lischer 2010). ARLEQUIN default parameters of 1,000,000 steps in the Markov Chain and 100,000 dememorisation steps were used for HWE, and 10,000 permutations for LD. The critical *P*-

values were corrected for multiple comparisons using the Benjamini-Yekutieli (B-Y) correction (Narum 2006). The B-Y correction is a more moderate correction than the commonly-used Bonferroni correction, providing a more conservative Type I error rate. As such, it is recommended for use in conservation genetic studies, allowing more consistent detection of significance, thereby improving statistical power (Narum 2006).

2.4 Results

Of the 9,596,798 raw reads produced from the Illumina MiSeq 250 bp paired-end run, 50,795 were identified by PAL_finder as containing potential microsatellites. Primers were designed for 10,126 of these reads; those that contained sufficient overlap to be collapsed. These 10,126 reads consisted of 5,766 reads containing one or more dinucleotide repeats, 2,246 reads containing one or more trinucleotide repeats and 2,114 reads containing one or more tetranucleotide repeats.

From these reads, forty loci were selected for testing, 27 of which amplified successfully and consistently, producing a single band in black robins (see Table 2.1). These 27 loci were genotyped using a combination of individuals from Rangatira and Mangere islands. Of these, 12 loci were monomorphic, and a further four proved difficult to score due to stutter or multiple peaks, and these were all removed from the final set. In total, 11 loci were polymorphic in black robins.

Of the 27 loci that amplified in black robins, only PT15 failed to amplify in tomtits. All 26 remaining loci were genotyped. Loci were more frequently polymorphic, and fewer individuals were required to determine polymorphism than in the black robin. Of the 26 loci, 17 were polymorphic in tomtits, including eight that were polymorphic in both species.

Table 2.1 Results of testing for amplification and polymorphism of forty microsatellite loci isolated from the black robin**.** GenBank accession numbers KU194428-KU194467. n = number of individuals genotyped, T_A = annealing temperature (T_A in bold represents final T_A used), Result = result of genotyping, TD = touchdown protocol, D = did not amplify well and could not be genotyped, P = polymorphic, M = monomorphic at a single allele, SD = scoring difficulties due to stuttering.

					Black robins		**Tomtits**	
Locus	**Forward primer**	**Reverse primer**	**Repeat motif**	**TA (°C) tested**	**n**	**Result**	**n**	**Result**
PT1	GGCCCATCTTTGAAGGTTCT	TCCAAAGTATCCCACCAGCA	AAAG(23)	50, 52, **TD**	6	P	6	SD
PT10	CCTGCTCAAACTGAAGTCCG	TGACATCCCCTTTGTGATGC	TTC(20)_TCC(11)	52, **54**	8	P	6	P
PT11	ATTGTCTACTGGCAGCTGGA	AGGAAAGATAGAGCTAAATTCGC	AT(21)_AC(18)	47, 52, 56, 58, 62, TD	D	-	-	-
PT12	CACAGGGGAGGACTTGAGAA	AGTTCAGGTCTGCTTTGTACA	ATAC(11)_AT(15)	46, 47, 50, 52, **TD**	6	M	6	P
PT13	CGCAGACACCTCATTCCTGA	TGACTGCCTTTTCCACTGGA	ATC(31)	52, 56, 58, 62	D	-	-	-
PT14	CTTTCCCTGCTTCCTGTGC	TTCAGTGGGTTTGGGATGGA	ATGG(19)	52, 56, 58, 60, 62	D	-	-	-
PT15	CGGTTCAGCACGTGTTCC	GAACTACCAGCAGACAGGGT	ATT(22)	50, **52**, 54, 62	6	SD	D	-
PT16	GAGCTGCTCTATCACTGCCT	CCACAAAGGCATTCAGGGAG	TC(28)	46, 48, 50, 52	D	-	-	-
PT17	TCATTCGAGACTCCATTCCT	TCGAGAAGGAGGCGAATGAA	TGC(20)_ATT(11)	52, 56, 58, 62, TD	D	-	-	-
PT18	TCCCACCCTGTTCTCCATTT	TTAGGTGCCGTGACTTCTCA	AGT(21)	52, **56**	6	P	7	P
PT19	AGCAGGCATTTTCAGCACTC	GCAACCCCACAAAACCTGAT	AGTG(17)	52, **56**	6	M	8	P
PT2	GCTATTTGTGCAGGGAAGTG	TGTGCCAGATTTTCCACAGC	AAAG(23)	50, 52, 54, **56**	8	P	6	P
PT20	GAGCTGCCACTGGTGTAAAG	GGCAGGTGAATGTCTTAGTT	AT(20)	46, 48, 52, **TD**	6	SD	6	M
PT21	ATCCTTCCCTTAGCCTGGTG	TCCAACAACAGTGCAGTTCA	AT(24)	47, 48, **50**, 52	6	SD	6	M
PT22	AGGACAATTCAACCTCCCTGT	TCCCAAAATGTTTCACAGGT	AT(26)	45, 46, 48, 50, 52, TD	D	-	-	-
PT23	ACATAATGGGTGCAACTGGG	ACATCCAAATACTTGCATGGGG	AT(27)	50, 54, **TD**	7	SD	9	SD
PT24	GCCAGCAGGTAAGTTGTGCT	CCTCCCTTCTCCCTGTCTCT	ATC(24)	50, 52, 55, 56, 58, **60**, 62	7	M	6	P
PT25	TAAAGGGAGCAAAGGAGGCA	TGTCCAAAGTCCCTCTCCAG	ATGG(17)	52, 54, **58**	11	M	11	P
PT26	TGCCATGCTTATTCTGGGGA	CTATGGGAGTGTGCCTGTAG	ATGG(17)	52, **TD**	12	P	14	P
PT27	ACATACCACCTGCCACTTCA	TGTCTGGGCTTTAATGTCTCAC	ATT(28)	52, 55, 56, 58, **60**, 62	11	P	6	P

Table 2.1 continued

Locus	Forward primer	Reverse primer	Repeat motif	TA (°C) tested	Black robins		Tomtits	
					n	Result	n	Result
PT28	GATGAGAATGTGCCTGCCTG	TCTGCAGCAAGAACACAACA	ATT(21)	47, 50, 52, 54	D	-	-	-
PT29	CAAACTGAAAGCTCCAAAGGG	TGATCTCGCAGGTAATTCTCC	ATT(22)	50, 52, 58, TD	D	-	-	-
PT3	CTCCAAGGCACTCTCTTTTCC	ATGTAACCAGGGCAGATGGC	AAAT(16)	46, 48, 50, **54**	8	SD	7	SD
PT30	TTCCTCCAACAGATCAAAGT	AGGAAGTTTTACTGAGGCAA	ATT(24)	45, 46, 48, 50, 52	D	-	-	-
PT31	TCTGTGCTGTGCCCTGAG	TATTCCTCTCGTTTCCCCGG	TGC(19)	52, 54, **TD**	8	SD	6	SD
PT32	CGTTAGATTTGCACATTGGCA	AGACCAACTCCACAGCTCC	TTC(27)	52, 54, **56**	6	M	8	M
PT33	CCCTCTTATGCCCAAAAGCC	GGTTTTGTAAAGGCAAGCAAGG	TTGG(15)	50, 52, 54, TD	D	-	-	-
PT34	TTGAAGCTGTCCCTTTCCCT	CTGTTCTTCCTCAGTTTTATGGA	ATT(31)	47, 50, 52, 58, TD	D	-	-	-
PT35	CTGAAAGAGGGCACAGCTTC	GAGATGCACTTCTTTGGCGA	AC(20)	52, **56**, 60, 62	6	M	8	P
PT36	GCTGTGAGGTTTGTGTGCTT	ACAGAGTCATTTAGTGTACAGCA	AC(20)	47, 50, **52**, 54, TD	D	-	-	-
PT37	TCTTGGTGGGGATCTACACAC	ACTTCCCATGGCAGAACAGT	AC(21)	52, 56, **58**, 60, 62	6	P	6	P
PT38	CCTGCCCAGACCAACTCT	AAATGAATCCTCGCTGTCCA	AC(21)	47, 50, 52, 54, **TD**	7	P	6	M
PT39	CCATGCAACTACGGGTGTTT	TGTCTGAGAACCCCAGAAGG	AC(21)	**48**, 50, 52	13	P	6	P
PT4	TATCTCCACATGGTGCAGGC	TGCTATGGTTTTATGCCCTGG	AATG(14)	52, **54**	8	M	6	P
PT40	ACTTTGAAATACTCTCGAGGGC	TGGAATCCATTTTGTGCAAT	AC(25)	**48**, 50, 52, 54	6	P	6	M
PT5	GTCTCTGGTGAGTCCTGGGG	GGGGTTTGGAACAATCATCC	AGGG(10)_ATGG(10)	50, 52, **54**	6	SD	6	P
PT6	CAACCATGTGAACGGTCTGC	AAAGGAGTGGGATTTGGAAGC	AGT(21)	52, 53, **54**	6	M	6	P
PT7	GCTCACCTTTTACAATCCTCTGC	CCTGCTGCTGTTTAGAAGCC	ATCT(15)	50, 52, 53, **54**	12	P	6	P
PT8	TTTTGGGTGCTCAACACTGG	TCTCTGGGAGAGAAGCCACC	ATCT(17)	48, 50, 52, 54	D	-	-	-
PT9	CCTCTTGGAGAGGTTCTGCG	GTGATGAGTCAGCTCCAGCG	TGC(23)	48, 50, 54, **TD**	6	M	8	P

The maximum number of alleles per locus was three in black robins (average ± SE = 2.273 ± 0.141), and ten in tomtits (average ± SE = 4.471 ± 0.529; Table 2.2). For the eight loci polymorphic in both species, the maximum number of alleles per locus was three in black robins (average ± SE = 2.25 ± 0.164) and seven in tomtits (average ± SE = 3.875 ± 0.693).

Table 2.2 Characterisation of twenty microsatellite loci developed for the Chatham Island black robin and cross-amplified in the Chatham Island tomtit. T_A = optimal annealing temperature, bp = base pairs, n = number of individuals genotyped, N_A = number of alleles, H_O = observed heterozygosity, H_E = heterozygosity expected under Hardy-Weinberg Equilibrium (values in bold indicate significant deviation from HWE), M = monomorphic, D = did not amplify consistently.

			Black robins				**Tomtits**			
Locus	**T_A (°C)**	**Allele size range (bp)**	**n**	**N_A**	**H_O**	**H_E**	**n**	**N_A**	**H_O**	**H_E**
PT1	TD	322-360	30	3	0.567	0.660	8	D	-	-
PT10	54	325-338	30	2	0.300	0.381	30	2	0.400	0.325
PT12	TD	192-198	8	M	-	-	30	4	0.567	0.610
PT18	56	220-229	30	2	0.467	0.364	30	3	0.600	0.677
PT19	56	173-201	7	M	-	-	30	7	0.833	0.812
PT2	56	202-226	30	2	0.367	0.305	30	6	0.700	0.754
PT24	60	119-153	9	M	-	-	29	3	0.200	**0.640**
PT25	58	150-208	8	M	-	-	29	10	0.586	0.727
PT26	TD	196-238	30	2	0.033	0.033	30	4	0.467	0.681
PT27	60	214-240	30	3	0.300	0.362	28	7	0.714	0.690
PT35	56	83-88	8	M	-	-	30	3	0.467	0.453
PT37	58	121-141	30	2	0.300	0.305	30	2	0.400	0.506
PT38	TD	101-105	30	2	0.233	0.259	8	D	-	-
PT39	48	99-109	30	2	0.367	0.463	30	2	0.367	0.345
PT4	54	281-300	8	M	-	-	29	6	0.897	0.753
PT40	48	132-143	30	2	0.557	0.508	8	M	-	-
PT5	54	246-254	8	M	-	-	30	3	0.433	0.515
PT6	54	268-300	8	M	-	-	30	5	0.467	0.563
PT7	54	265-288	30	3	0.600	0.594	30	5	0.800	0.755
PT9	TD	285-304	8	M	-	-	29	4	0.300	**0.488**

Testing with MICROCHECKER revealed no evidence of null alleles. PT9 and PT2 significantly deviated from HWE in tomtits (B-Y corrected $P = 0.0145$; Table 2.2), while no loci deviated from HWE in black robins. Significant LD was detected in three loci pairs in tomtits (PT18 and PT26, PT39 and PT27, and PT6 and PT24; B-Y corrected $P = 0.0091$), but was not detected for any loci in black robins (B-Y corrected $P = 0.0109$). As significant LD was not detected for the same loci pairs in both species, they are unlikely to be physically linked. Average expected heterozygosity over the eight loci polymorphic in both species was 0.351 ± 0.056 in black robins and 0.592 ± 0.062 in tomtits. Only two of the eight loci had greater expected heterozygosity in black robins than tomtits (PT10 and PT39).

2.5 Discussion

The combination of relatively low microsatellite frequency in birds (Primmer *et al.* 1997), and the expected low level of variation in the black robin due to a high level of inbreeding was expected to result in a low percentage of polymorphic loci being detected. However, we successfully detected 11 polymorphic loci for black robins, out of forty loci tested. This demonstrates that Illumina sequencing is extremely useful for studies of similar populations with low diversity, as it provides a much greater number of microsatellite sequences to select from compared to the traditional enriched library, or 454 sequencing, for a similar cost (Abdelkrim *et al.* 2009; Castoe *et al.* 2012). As such, many loci can be selected with preferable characteristics, such as long repeat motifs for ease of scoring, and higher repeat numbers that are more likely to be polymorphic (Castoe *et al.* 2012) which is very useful for study populations with low variability. Additional loci could have been added to the total of polymorphic loci, either by a) selecting a greater number of microsatellites to design primers for, or b) by adjusting the primer sequences that amplified poorly or inconsistently for the loci that were excluded prior to genotyping, as for PT24 and PT27. However, the number of loci developed here exhibit sufficient variation and statistical power to answer questions relating to overall species diversity and population differentiation.

While diversity is typically lower in non-target species due to ascertainment bias (Galbusera *et al.* 2000; Primmer *et al.* 1996), it is likely to have little effect in this comparison due to how closely related these sister-species are (Miller & Lambert 2006). Testing showed the black robin displayed lower diversity than the tomtit across almost all shared loci in the

populations sampled. These results indicate that compared to the tomtit, genetic drift has likely had a strong effect on diversity due to the history of small population size and the extreme bottleneck experienced by the black robin.

The polymorphic microsatellite primers described here substantially increase the number of polymorphic loci known to amplify in black robins. Combined with the three previously-reported polymorphic loci (Cubrinovska *et al.* 2016), I am now able to assess genetic diversity and fine-scale population structure across the two island populations (see Chapter Three). The level of variation at these loci will also enable testing for extra-pair paternity in this socially monogamous species (see Chapter Four). Cross-species amplification of many of these loci in tomtits indicates their usefulness for other studies within the *Petroica* genus.

Chapter Three

Genetic diversity and population differentiation within and between two island populations of the Chatham Island black robin

3.1 Abstract

Small populations are more prone to extinction largely due to the effects of genetic drift and inbreeding reducing genetic variation and fitness of such populations. The Chatham Island black robin *Petroica traversi* is one of the world's most inbred species still surviving in the wild. Following a severe population bottleneck, the species has recovered to around 290 individuals on two isolated islands. Nevertheless, the severe bottleneck and subsequent intense inbreeding have likely had long-term consequences affecting the viability of this endangered species. In this chapter, I analysed the genetic diversity of the black robin, and found low levels of microsatellite diversity at eight polymorphic loci. Both the number of alleles and expected heterozygosity were lower in the black robin than in its sister-species, the Chatham Island tomtit *Petroica macrocephala chathamensis*, which coexists on Rangatira and Mangere islands. I also found that the two island populations of black robin have differentiated from one another, likely due to strong genetic drift acting independently on these populations over 26 years of isolation. Reciprocal translocations of individuals between islands is recommended to prevent further loss of diversity through drift, particularly in the smaller Mangere Island population, and so to improve the probability of species persistence.

3.2 Introduction

Small populations are inherently at a higher risk of extinction than larger populations due to demographic, environmental, and genetic stochasticity (Caughley 1994; Lande 1993). Genetic stochasticity (also referred to as genetic drift) is the chance fluctuation in allele frequencies caused by random sampling effects between generations (Lande 1988). These random fluctuations are much greater in small populations, and can substantially reduce genetic diversity as alleles become fixed at random (Lande 1988). Strong genetic drift in small populations may overwhelm the effects of natural selection, and may result in

deleterious alleles becoming fixed in the population, rather than being removed or maintained at low frequencies by natural selection (Keller & Waller 2002). The accumulation of deleterious mutations may reduce survival and reproduction, driving the population towards extinction (Lynch *et al.* 1995). In addition, in extremely small populations, relatives may have no alternative but to mate with each other, resulting in inbred offspring. Inbreeding leads to an increase in homozygosity, and therefore the increased expression of recessive deleterious alleles that results in reduced fitness of inbred progeny as compared to those of random matings (Keller & Waller 2002). This reduction in fitness is known as inbreeding depression, and can affect fitness traits at all life stages (Charlesworth & Charlesworth 1999; Grueber *et al.* 2010; Keller & Waller 2002; Wright *et al.* 2008). Decreased fitness of individuals reduces population growth, and eventually creates a positive feedback loop as the population becomes ever smaller and more inbred, accelerating the population towards extinction (Fagan & Holmes 2006). The combined effects of genetic drift and high rates of inbreeding can increase extinction vulnerability. Moreover, low genetic variation limits the adaptive potential of small populations, reducing their ability to adapt and survive if environmental conditions change (Caballero & García-Dorado 2013).

The Chatham Island black robin (*Petroica traversi*) is an endangered passerine endemic to the Chatham Islands, an archipelago 800 km east of New Zealand (BirdLife International 2016b; Massaro *et al.* 2013a). Approximately 35 individuals survived for over eighty years on a single small island (Little Mangere Island), but the black robin became known as the world's most endangered bird when in 1980 the population was further reduced to include only a single breeding pair (Butler & Merton 1992; Massaro *et al.* 2013a). Conservation management prevented extinction of the species by cross-fostering black robin eggs and nestlings to its morphologically similar sister-species, the Chatham Island tomtit (*Petroica macrocephala chathamensis*), which stimulated increased egg laying by the black robin (Butler & Merton 1992). Translocations during 1982 - 1990 relocated an estimated 23 birds and 53 eggs from Mangere to Rangatira (Hokorereora) Island (~11 km south-east of Mangere Island; see Figure 3.1) to establish a second population (Butler & Merton 1992; Kennedy 2009). Currently the species numbers around 290 individuals; with 247 robins on Rangatira and 43 on Mangere (M. Massaro, personal communication). Although the population size has recovered from the severe population bottleneck in 1980, high levels of inbreeding when the population was extremely small (Ardern & Lambert 1997) may have contributed to increase

population vulnerability, as some individuals are estimated to have inbreeding coefficients that are higher than those in selfing populations (Kennedy *et al.* 2014; Weiser *et al.* 2016). Severe inbreeding has led to reduced fitness, such as reduced juvenile survival (estimated at 6.85 lethal equivalents (Kennedy *et al.* 2014), and the impact of strong drift is exhibited in the spread of an odd mal-adaptive trait, whereby females lay eggs on the rim of their nests, which then fail to hatch as they are not incubated (Massaro *et al.* 2013a).

Figure 3.1 Map of Rangatira (South East) Island, depicting the northern Woolshed Bush, central Top Bush, and Skua Gully. Individuals north of the yellow line were defined as belonging to the 'Woolshed' population, while those to the south are designated as part of the 'Top Bush' population for the purpose of this study.

No dispersal of black robins between Mangere and Rangatira has ever been observed, with the limited dispersal capability of the black robin across open areas preventing movement between these two islands (Butler & Merton 1992). Hence both populations on Rangatira and Mangere islands will have independently experienced genetic drift during the 26 years of isolation, and so may have differentiated from one another. Drift is likely to be stronger in the very small Mangere population, and may have resulted in lower diversity in this population compared to Rangatira.

The single population on Mangere Island is assumed to be at carrying capacity, as slow forest regeneration is limiting population growth (Kennedy 2009). On Rangatira, the black robin is hypothesised to exist in two populations, each inhabiting a distinct bush area; Woolshed Bush to the north, and Top Bush to the south, separated by Skua Gully (see Figure 3.1) (Kennedy 2009; Weiser *et al.* 2016), where brown skuas (*Catharacta skua lonnbergi*) once nested at high densities (Butler & Merton 1992). The presence of skuas, coupled with an avoidance of open areas by black robins, may have limited dispersal between forest patches (Butler & Merton 1992; Kennedy 2009), and so there may be some level of differentiation between robins in these two distinct habitat areas.

Future aims for management of this endangered species include the establishment of additional populations on Little Mangere and Pitt islands to reduce extinction risk. An attempt was made to establish a third black robin population by translocating individuals to a predator-free fenced area (the Ellen Elizabeth Preece Conservation Covenant) on Pitt Island between 2002 – 2004 (Kennedy 2009). However, all 34 robins translocated had died or disappeared by the end of 2007 with no clear cause, and no further translocations have been attempted (Kennedy 2009). Assuming the existing island populations are genetically differentiated, another option to improve diversity within populations may be to conduct reciprocal translocations of individuals between islands, which may also reduce levels of inbreeding and improve fitness and population growth (Heber & Briskie 2013; Johnson *et al.* 2010).

In this chapter, I assess the levels of genetic diversity within and among the two black robin populations on Mangere and Rangatira, by using polymorphic microsatellite loci developed via next-generation Illumina sequencing from the Chatham Island black robin genome (see Chapter Two). A number of these markers amplify and are polymorphic in the Chatham Island tomtit (see Chapter Two). The Chatham Island tomtit co-exists with the black robin on Mangere (70 – 100 adults) and Rangatira islands (200 – 300 adults), with a third population of approximately 900 adult birds on Pitt Island (Department of Conservation [DOC], 2001b). Measuring diversity in the tomtit will allow comparison with a similar species that has not experienced such an extreme bottleneck and severe inbreeding as the black robin.

In combination with the markers designed for the black robin, I will also test twenty microsatellite loci designed for the South Island robin *Petroica australis* (Townsend *et al.* 2012), and use any polymorphic loci in this study, along with three further loci previously found to be polymorphic in the black robin (Cubrinovska *et al.* 2016), developed for the zebra finch (*Taeniopygia guttata*, locus TG02-088) (Dawson *et al.* 2010), red-capped robin (*Petroica goodenovii*, locus PGM1) (Dowling *et al.* 2003), and the North Island saddleback (*Philesturnus carunculatus rufusater*, locus PCA12) (Lambert *et al.* 2005). Using these markers, I will compare levels of diversity within the two black robin populations, and examine whether these populations have differentiated over the past 26 years of isolation. Additionally, I will determine whether the distinct forest patches on Rangatira house differentiated black robin populations.

The findings from this study will be used to assist the Department of Conservation (DOC) to develop future management plans for the iconic Chatham Island black robin, by making recommendations as to how best to manage the Rangatira population, and whether to conduct translocations to improve the future outlook for this endangered species.

3.3 Methods

3.3.1 Sampling and extraction

Black robin and tomtit samples were collected from Mangere and Rangatira each breeding season from 2008 to 2011, and also in 2014, using mistnets or drop traps to capture birds. Brachial venepuncture was used to collect blood samples that were stored in ethanol, lysis buffer, or dried on filter paper. Alternatively, feather samples were collected from some birds.

DNA was extracted from blood spots or feathers of black robin and tomtit individuals using an Invitrogen PureLink™ Genomic DNA Mini Kit. There were 250 samples previously extracted for a prior study (Cubrinovska *et al.* 2016), with an additional 71 black robin individuals sampled in 2010, 2011, and 2014 extracted for inclusion in this study. The mammalian tissue protocol was used for DNA extraction of blood stored in ethanol and feather samples, blood lysate protocol used for blood stored in lysis buffer, and dried blood spots on filter paper were extracted following the blood spot protocol (Invitrogen PureLink Genomic DNA kit user manual). DNA extraction concentrations were measured using a NanoDrop® ND-1000 spectrophotometer. Individuals with higher DNA concentrations (> 100 ng/µl) were initially selected for primer testing to ensure consistent amplification of microsatellite loci.

3.3.2 Individuals selected for genotyping

Thirty black robin individuals from Mangere were preferentially selected for genotyping based on DNA quality at extraction, to ensure amplification of loci. These thirty individuals comprised more than half the total current population of approximately fifty individuals. Pedigree information was not available for Mangere, and so sampled individuals were assumed to be non-familial.

From the Rangatira black robin population, 174 individuals were genotyped in total. A much larger number of individuals from the Rangatira population were genotyped to allow assessment of extra-pair paternity (see Chapter Four). However, closely related individuals are more likely to have similar allele frequencies than the true population mean, and so only a single individual from each known family group (including observed parents, offspring, or siblings) was included in the analyses of diversity and differentiation to limit bias in mean

allele frequencies (n = 115). All 204 individuals genotyped (Mangere n = 30, Rangatira n = 174) were included in testing to identify potential dispersal between islands.

To investigate population subdivision within Rangatira, individuals were separated into 'Woolshed Bush' (north of the yellow line) and 'Top Bush' (south of the yellow line) populations (see Figure 3.1), based on GPS data from natal or breeding locations. These locations were recorded by GPS (Garmin GPSMAP60CSx, < 10 m) during banding and sampling of individuals. Of all samples available, only 27 were determined to belong to the Top Bush population, 25 of which were genotyped and included in this analysis. These 25 were compared against a random subset of thirty individuals from the Woolshed population. As demonstrated in Hale *et al.* (2012), 25 to thirty individuals is sufficient to estimate the level of diversity within a population, as increasing costs outweigh the smaller gains in information.

Tomtit individuals from both Mangere (all available samples included, n = 22) and Rangatira (n = 30, randomly selected from 52 available samples) were selected for genotyping to allow for comparisons of diversity with the black robin.

3.3.3 Microsatellite markers

All 11 loci polymorphic in black robins and 17 loci polymorphic in tomtits developed in Chapter Two were used here. Loci were amplified in 15 µl reactions containing 0.5 µl of genomic DNA, 1 x NH_4 reaction buffer (Bioline), 2 mM $MgCl_2$, 0.08 mM dNTPs, 0.083 µM of forward primer, 0.33 µM reverse primer and 0.33 µM fluorescently labelled M13 primer (5'-TGTAAAACGACGGCCAGT, labelled with 6-FAM, NED, VIC, or PET; Applied Biosystems), and 0.6 U BIOTAQ DNA polymerase (Bioline). For PT2 and PT27, $MgCl_2$ was increased to 2.33 mM per reaction to improve annealing. An Eppendorf® Mastercycler® was used for PCR thermocycling. One of two thermocycling protocols was used. The standard three-step thermocycling protocol consisted of: 95°C for 12 min, 10 cycles of 94°C for 15 s, annealing temperature (T_A) °C for 30 s, 72°C for 30 s, followed by 30 cycles of 89°C for 15 s, T_A°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 10 min (see Table 3.1 for annealing temperatures).

Table 3.1 List of microsatellite loci used for genotyping in each species. T_A = final annealing temperature used, ✓ indicates this locus was used in genotyping of black robins and/or tomtits in this study

Locus	T_A (°C)	Black robins	Tomtits
PT1	TD1	✓	
PT10	54	✓	✓
PT12	TD1		✓
PT18	56	✓	✓
PT19	56		✓
PT2	56	✓	✓
PT24	60		✓
PT25	58		✓
PT26	TD1	✓	✓
PT27	60	✓	✓
PT35	56		✓
PT37	58	✓	✓
PT38	TD1	✓	
PT39	48	✓	✓
PT40	54	✓	
PT4	48		✓
PT5	54		✓
PT6	54		✓
PT7	54	✓	✓
PT9	TD1		✓
PAU26	48	✓	
PCA12	64	✓	
PGM1	56	✓	
TG02-088	50	✓	

When initial $T_A \geq 54$°C, the protocol was altered to 95°C for 12 min, 10 cycles of 94°C for 15 s, T_A°C for 30 s, 72°C for 30 s, followed by 30 cycles of 89°C for 15 s, 53°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 10 min. This improved annealing of the M13 primer (Schuelke 2000), except with PT2, PT24, and PT27, where the original protocol was

used. For primers that failed to amplify consistently at a single annealing temperature, a touchdown sequence (TD1) was used, comprising: 95°C for 15 min, 10 cycles of 94°C for 15 s, T_A°C (starting at 62°C and decreasing 1°C per cycle over ten cycles) for 30 s, and 72°C for 1 min, then 25 cycles of 94°C for 15 s, 52°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 15 min. PCR products were visualised by electrophoresis on a 1.4% agarose gel pre-stained with SYBR® Safe to ensure consistent successful amplification.

In combination with the loci developed in Chapter Two, twenty loci developed for the South Island robin (Townsend *et al.* 2012) were tested for amplification and polymorphism in black robins (see Table 3.2). All loci were initially tested for amplification in 15 µl reactions containing 0.5 µl of genomic DNA, 1 x NH_4 reaction buffer (Bioline), 2 mM $MgCl_2$, 0.08 mM dNTPs, 0.33 µM of forward primer, 0.33 µM reverse primer, and 0.6 U BIOTAQ DNA polymerase (Bioline). The touchdown thermocycling protocol (TD2) described in Townsend *et al.* (2012) was used, which consisted of: 95°C for 15 min, a touchdown sequence comprising 94°C for 30 s, T_A (starting at 60°C and decreasing 1°C per cycle over eight cycles) for 90 s and extension at 72°C for 60 s, followed by 25 cycles at 94°C for 30 s, 52°C for 90 s and 72°C for 60 s and a final 30 min hold at 60°C. Where loci did not amplify successfully using this protocol, the standard three-step cycle stated above was used, with a range of T_A tested. For all loci that amplified consistently, fluorescently labelled M13 primers were added in 15 µl reactions containing 0.5 µl of genomic DNA, 1 x NH_4 reaction buffer (Bioline), 2 mM $MgCl_2$, 0.08 mM dNTPs, 0.083 µM of forward primer, 0.33 µM reverse primer and 0.33 µM fluorescently labelled M13 primer (one of 6-FAM, NED, VIC, or PET, Applied Biosystems), and 0.6 U BIOTAQ DNA polymerase (Bioline), and loci were re-amplified for genotyping. At least eight black robin individuals were genotyped for all loci that amplified consistently to test for polymorphism. Samples were prepared for genotyping by adding 0.5 µl PCR product to 0.3 µl Genescan 500LIZ size standard (Applied Biosystems) and 12 µl HiDi formamide. These were then denatured at 95°C for five minutes. Genotyping was performed on an ABI Prism® 3130xl Genetic Analyser (Applied Biosystems). Allele sizes were scored visually using GeneMarker (v.2.20; SoftGenetics). From this set of twenty loci, nine loci amplified poorly or inconsistently, and ten appeared monomorphic (see Table 3.2). The sole polymorphic locus, PAU26, was incorporated in the final set used here (see Table 3.1).

Table 3.2 Results of testing for amplification and polymorphism of twenty loci developed for the South Island robin (Townsend *et al.* 2012). T_A = range of annealing temperatures tested, values in bold represent the most successful T_A.

Locus	**T_A tested (°C)**	**Result of amplification**	**Result of genotyping**
PAU1	48, TD2	Did not amplify	-
PAU2	**TD2**	Amplified	Monomorphic
PAU4	**TD2**	Amplified	Monomorphic
PAU6	50, TD2	Amplified inconsistently	-
PAU7	**TD2**	Amplified	Monomorphic
PAU8	**TD2**	Amplified	Monomorphic
PAU9	**46**, 48, 50, TD2	Amplified	Monomorphic
PAU16	48, **50**, TD2	Amplified	Monomorphic
PAU17	50, TD2	Amplified inconsistently	-
PAU24	**TD2**	Amplified	Monomorphic
PAU25	48, TD2	Did not amplify	-
PAU26	48, **50**, TD2	Amplified	Polymorphic
PAU28	62, TD2	Amplified	Monomorphic
PAU39	62, TD2	Amplified inconsistently	-
PAU63	46, 48, 50, 62, TD2	Amplified inconsistently	-
PAU66	**TD2**	Amplified	Monomorphic
PAU67	**TD2**	Amplified	Monomorphic
PAU77	48, 50, TD2	Did not amplify	
PAU81	TD2	Amplified inconsistently	-
PAU82	TD2	Amplified inconsistently	-

A further three polymorphic loci, TG02-088, PCA12, and PGM1, that were previously found to be polymorphic in black robins were also included in this study for black robin genotyping (Cubrinovska *et al.* 2016; Dawson *et al.* 2010; Dowling *et al.* 2003; Lambert *et al.* 2005). Forward primers were labelled with a fluorescent dye (6-FAM, NED, or PET, Applied Biosystems). Loci were amplified in 15 µl reactions containing 0.5 µl of genomic DNA, 1 x NH_4 reaction buffer (Bioline), 2 mM $MgCl_2$ (decreased to 0.167 mM for PGM1 to improve annealing), 0.08 mM dNTPs, 0.33 µM of forward primer, 0.33 µM reverse primer, and 0.6 U

BIOTAQ DNA polymerase (Bioline). All three loci were amplified using the standard three-step PCR protocol initially described (see Table 3.1 for annealing temperatures).

To minimise genotype scoring error and test for contamination, a negative control was incorporated in every PCR amplification, consisting of the reaction mix without DNA added. When a different fluorescent dye was added to the reaction mix than had been previously used, at least one positive control was included. This used a sample that had been genotyped previously, to confirm accuracy of scoring even if there was some difference in allele size due to the different dye. Differences in allele sizes with different fluorescent dyes were clear and consistent.

The final set of loci used comprised 15 loci in black robins and 17 in tomtits (see Table 3.1). Individuals with missing data at more than two loci were excluded from the subsequent tests. To measure genotyping error that may result in incorrect identification of genotypes and estimates of allele frequencies (Bonin *et al.* 2004; Broquet & Petit 2004), 14.2% of samples per locus were amplified, genotyped, and scored twice. The error rate was calculated by dividing the number of errors by the total number of samples repeated (Hoffman & Amos 2005).

3.3.4 Statistical analysis

STRUCTURE ver. 2.3.4 (Pritchard *et al.* 2000) was used to estimate the number of true genetic clusters, firstly to clarify differentiation between Mangere and Rangatira populations for both species, and secondly to investigate fine-scale structuring within the different forest areas in the Rangatira black robin population. Using the given data, K, the true number of genetic clusters, was estimated using Bayesian clustering (Pritchard *et al.* 2000). A burn-in length of 10,000 followed by 100,000 iterations was used to produce consistent results in replicate runs. The admixture model using LOCPRIOR and correlated allele frequencies was used, taking into account the sampling locations (either Mangere or Rangatira, or Woolshed or Top Bush). This is a more informative method when weak population structuring is expected (Porras-Hurtado *et al.* 2013), which is likely given the history of small population size, intense inbreeding, and translocations experienced by the black robin. Each of the tomtit

(n = 52) and black robin data sets (n = 115) were analysed with K = 1 - 3 to test for between-island differentiation, allowing identification of potential localised structuring within islands. The subset of black robins from separate bush areas on Rangatira was run separately with K = 1 - 2 to determine the presence of any substructuring between individuals in the two forest areas. Analysis for each value of K was repeated twenty times to obtain means and standard errors. Results were visualised using STRUCTUREHARVESTER ver. 0.6.94 (Earl & von Holdt 2012). Comparison of mean log-likelihoods and variance of the range of K values was used to determine the most likely number of clusters present, with the highest value indicating the most likely K. In this situation, the commonly used ΔK method (Evanno *et al.* 2005) is not appropriate, as it cannot detect when true K = 1, which is a possibility that cannot be ruled out for these populations. Independent runs were combined using CLUMPP ver. 1.1.2 (Jakobsson & Rosenberg 2007) and visualised with DISTRUCT ver. 1.1 (Rosenberg 2004).

All loci were tested for the presence of null alleles using MICROCHECKER ver. 2.2.3 (Van Oosterhout *et al.* 2004), with a confidence interval of 95% and 10,000 randomisations. Tests for deviations from Hardy-Weinberg equilibrium (HWE) and significant linkage disequilibrium (LD) were performed using ARLEQUIN version 3.5 (Excoffier & Lischer 2010). ARLEQUIN default parameters of 1,000,000 steps in the Markov Chain and 100,000 dememorisation steps were used for HWE, and 10,000 permutations for LD. These tests were corrected for multiple comparisons using the Benjamini-Yekutieli (B-Y) correction to provide a more conservative Type I error rate that is more appropriate for conservation genetic studies (Narum 2006). Each island population was tested separately. HWE and LD were also calculated for males and females of each species separately on each island to indicate whether sex linkage was likely.

Genetic diversity was quantified by calculating the allele frequencies using GENALEX ver. 6.5 (Peakall & Smouse 2006; Peakall & Smouse 2012), rarefied number of alleles using HP-RARE version 1.0 (Kalinowski 2005) to standardise for variation in sample sizes (Leberg 2002), and expected and observed heterozygosities using GENALEX (Peakall & Smouse 2006; Peakall & Smouse 2012).

To test for potential effects of different sample sizes on measures of diversity, the number of alleles, rarefied number of alleles, and expected and observed heterozygosities were calculated for a random subset of thirty Rangatira black robin individuals. The estimates of genetic diversity for this subset were then compared to the level of diversity measured across the larger data set.

Population differentiation within and among island populations was analysed by calculating F_{ST} and F'_{ST} (F_{ST} standardised for within-population variance; Hedrick 2005) in GENALEX (Peakall & Smouse 2006; Peakall & Smouse 2012) using 9999 permutations. While R_{ST} is commonly used to assess population structure in microsatellite-based studies, it is most useful for highly variable loci with many alleles, and is also recommended for use only with very large sample sizes (Meirmans & Hedrick 2011). The low level of variation and small sample sizes used here made the use of R_{ST} inappropriate for this study. F_{ST} was also calculated between the random subset of thirty black robin individuals from Rangatira and the Mangere population to assess whether there was any effect of sample size on differentiation between the two islands.

STRUCTURE ver. 2.3.4 (Pritchard *et al.* 2000) was used to test for the presence of dispersers in both tomtit and robin populations, with island populations predefined as independent clusters (K = 2), using a burn-in of 10,000 followed by 100,000 iterations. This was repeated ten times for each species to obtain mean values. Although no black robin dispersers have been detected from observations, it is important to determine whether any dispersal events have occurred that may allow natural gene flow between these islands. While hatching locations of black robins are available for some individuals from Rangatira Island during the 2007 – 2011 breeding seasons, all individuals (204 black robins, 52 tomtits) were included here regardless of known hatching location. Only a subset of individuals were sampled in each year, so inclusion of individuals regardless of hatching location may allow detection of individuals descended from dispersing individuals when samples from parents were unavailable. STRUCTURE identifies dispersers or their descendants as individuals having a low (< 50%) probability of being from the assumed population.

3.4 Results

3.4.1 Microsatellite loci

The final set of microsatellite loci used to genotype black robins included 11 polymorphic loci described in Chapter Two, one polymorphic locus developed for the South Island robin (PAU26), and three loci described for other species (PGM1, PCA12, and TG02-088). The genotyping error rate was low, estimated at 1.42%.

3.4.2 Population Structure

The Bayesian STRUCTURE analysis identified the presence of two distinct clusters among both black robins and tomtits (Table 3.3, Figures 3.2 and 3.3). In both species, all Mangere individuals formed one cluster, and all Rangatira individuals formed a second cluster. No evidence of structuring was detected within the Rangatira robin population (most likely K = 1), with all robins clustering as a single group, irrespective of the bush areas they inhabited (see Table 3.3).

3.4.3 Hardy-Weinberg equilibrium and linkage disequilibrium

After B-Y correction, one locus, PT27, significantly deviated from HWE in the Rangatira black robin population (B-Y corrected $\alpha = 0.0151$, $P = 0.0097$). No loci showed significant deviation in the Mangere black robin population. In tomtits, PT9 and PT24 significantly deviated from HWE in the Rangatira population (B-Y corrected $\alpha = 0.0145$, $P = 0.0007$ and ≤ 0.0001 respectively), but not on Mangere. No loci deviated from HWE in the subsample of thirty black robins from Rangatira.

Table 3.3 Results of cluster analysis. Means and standard deviations were calculated using STRUCTUREHARVESTER based on the results of STRUCTURE runs. K = number of clusters. Values in bold represent the most likely true K.

	K	Repeats	Mean LnP(K)	Stdev LnP(K)
Black robins (n = 145)	1	20	-2704.64	0.059
	2	**20**	**-2578.45**	**0.376**
	3	20	-2646.71	59.907
Rangatira black robins (n = 108)	**1**	**20**	**-1939.18**	**0.145**
	2	20	-2022.69	28.418
Tomtits (n = 90)	1	20	-1936.17	0.491
	2	**20**	**-1799.57**	**1.910**
	3	20	-1893.74	32.680

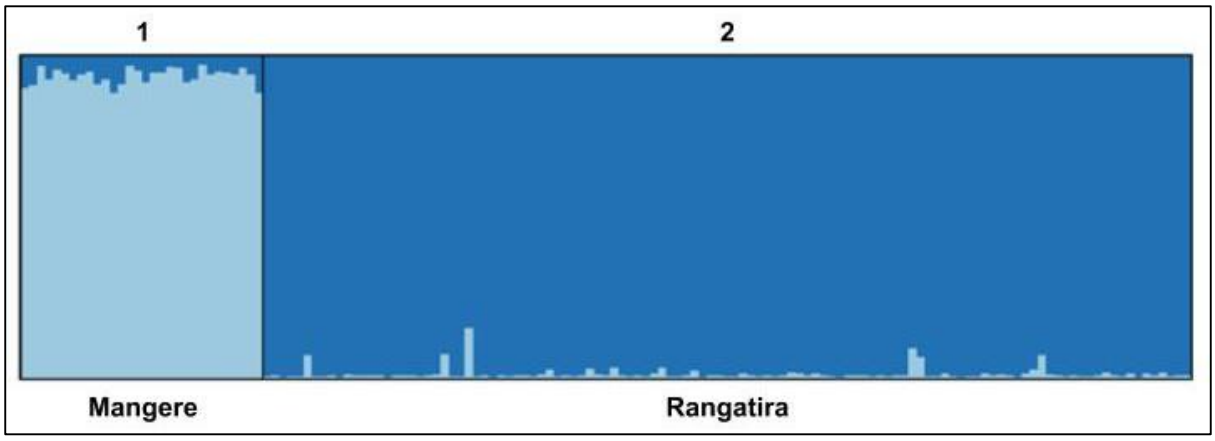

Figure 3.2 DISTRUCT visualisation of clustering in black robins. Each individual (n = 145) is represented by a vertical bar partitioned into 2 coloured segments according to the proportion of membership in each cluster (K = 2).

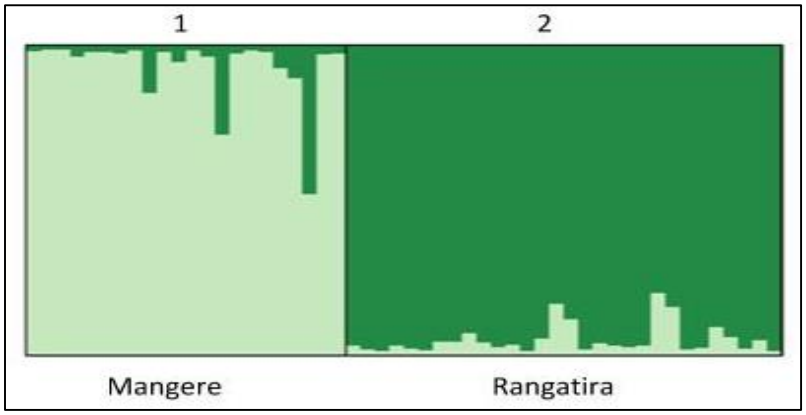

Figure 3.3 DISTRUCT visualisation of clustering in tomtits. Each individual (n = 90) is represented by a vertical bar partitioned into 2 coloured segments according to the proportion of membership in each cluster (K = 2).

In the black robin, 14 pairs of loci exhibited significant linkage disequilibrium on Rangatira (B-Y corrected $\alpha = 0.0096$), while eight pairs showed LD on Mangere. However, only one pair, PT1 and TG02-088, appeared significantly linked in both populations (see Table 3.4). Significant linkage was also found in black robin females in one pair of loci in both populations (PT2 and PT10), and in males in two pairs of loci in both populations (PT39 and PT40, and PT1 and TG02-088).

Significant LD was found in three pairs of loci in the Rangatira tomtit population (B-Y corrected $\alpha = 0.0091$), and in nine pairs in the Mangere tomtit population. Two of these pairs were found in both populations (see Table 3.4). In tomtits, two pairs appeared linked in males on both islands (PT39 and PT27, and PT6 and PT24). One pair of loci displayed significant linkage in both the black robin and the tomtit (PT39 and PT27). However, this pair did not appear significantly linked in the Mangere black robin population.

Analysis of separate populations with MICROCHECKER found an excess of homozygotes suggesting null alleles may be present at PT39 in the Rangatira black robin population, with significant null allele estimates of 0.092 (Van Oosterhout *et al.* 2004), 0.111 (Chakraborty *et al.* 1992), and 0.057 (Brookfield 1996).

While a substantial number of loci appeared linked in the black robin, the very low number that appeared linked in both populations or both species indicates that there are few pairs likely to be physically linked, with many likely to appear linked by chance. AMOVA analyses for the black robin were calculated without PT39 and PT27, and without PT27, PT10, and TG02-088. Analysis of tomtits were also carried out with and without PT9, PT24, and PT39. There was no obvious difference in results, and all loci were used in the subsequent analyses to increase statistical power.

3.4.4 Genetic diversity

Comparisons of allelic diversity and heterozygosity indicate that tomtits have greater diversity than black robins. For example, the number of alleles in black robins ranged from two to five across all 15 polymorphic loci (average = 2.53 alleles per locus; see Table 3.6; for allele frequencies see Appendix 2), while in tomtits this ranged from two to ten across 17 polymorphic loci (average = 4.47 alleles per locus; see Table 3.5; for allele frequencies, see Appendix 3). Tomtits displayed substantially higher variation than black robin for all measures at the eight shared polymorphic loci (see Table 3.7). In tomtits, 14 of 17 loci (82.4% of loci) contained more than two alleles, compared to the black robin where six of 15 loci (40%) had more than two alleles (see Tables 3.5 and 3.6). The Mangere populations of both species displayed generally lower diversity compared to Rangatira populations in terms of number of alleles and expected heterozygosity for most loci (see Tables 3.5 and 3.6). In the Rangatira black robin population, five alleles were present at locus PT1. When diversity measures were calculated for the random subset of thirty individuals from Rangatira, the number of alleles was higher in the larger sample, but there was little difference between the two data sets in terms of rarefied numbers of alleles and expected heterozygosities (see Table 3.6).

Table 3.4 Pairs of loci that displayed significant linkage disequilibrium in the Chatham Island black robin and the Chatham Island tomtit. Pairs of loci in italics indicate significant linkage in both populations of that species, while pairs of loci in bold represent significant linkage across species. *P*-values were calculated in ARLEQUIN.

Chatham Island black robin				**Chatham Island tomtit**			
Rangatira		**Mangere**		**Rangatira**		**Mangere**	
Loci	***P*-value**	**Loci**	***P*-value**	**Loci**	***P*-value**	**Loci**	***P*-value**
PT18 and PT7	< 0.0001	PT18 and PT2	0.0059	PT18 and PT26	0.0084	PT37 and PT7	0.0048
PT37 and PT39	0.0030	PT2 and PT10	0.0001	***PT39 and PT27***	<0.0001	PT37 and PT39	0.0050
PT39 and PT27	< 0.0001	PT18 and PT27	0.0077	*PT6 and PT24*	< 0.0001	***PT39 and PT27***	< 0.0001
PT37 and PT40	0.0080	PT10 and PT27	< 0.0001			PT18 and PT19	0.0020
PT39 and PT40	< 0.0001	PT26 and PT1	0.0007			PT37 and PT5	0.0011
PT40 and PT1	0.0037	PT18 and TG02-088	0.0055			PT39 and PT35	0.0083
PT2 and PAU26	0.0004	*PT1 and TG02-088*	0.0004			PT27 and PT35	0.0039
PT10 and PAU26	0.0013		< 0.0001			PT37 and PT25	0.0054
PT1 and TG02-088	< 0.0001					*PT6 and PT24*	< 0.0001
PT26 and PCA12	0.0023						
PT40 and PCA12	0.0023						
PT26 and PGM1	0.0041						
PT40 and PGM1	< 0.0001						
PCA12 and PGM1	< 0.0001						

Table 3.5 Allelic diversity and heterozygosity of tomtit populations on Rangatira and Mangere islands. n = number of individuals sampled, nA = number of alleles per locus, AR = allelic richness per locus (in terms of rarefied number of alleles), H_O = observed heterozygosity, H_E = heterozygosity expected under Hardy-Weinberg equilibrium, SE = standard error. H_E values in bold represent significant deviation from HWE.

	Rangatira					**Mangere**				
Locus	**n**	**nA**	**AR**	**H_O**	**H_E**	**n**	**nA**	**AR**	**H_O**	**H_E**
PT37	30	2	2.00	0.400	0.506	22	2	1.63	0.000	0.089
PT18	30	3	3.00	0.600	0.677	22	3	2.93	0.636	0.601
PT2	30	6	4.48	0.700	0.754	22	4	3.87	0.591	0.730
PT26	30	4	3.82	0.467	0.681	22	4	3.02	0.591	0.574
PT7	30	5	4.25	0.800	0.755	22	4	3.62	0.773	0.665
PT10	30	2	1.99	0.400	0.325	22	2	2.00	0.500	0.485
PT39	30	2	1.99	0.367	0.345	22	2	2.00	0.364	0.406
PT27	28	7	5.28	0.714	0.690	22	2	2.00	0.318	0.426
PT19	30	7	5.58	0.833	0.812	22	5	4.79	0.818	0.745
PT5	30	3	2.64	0.433	0.515	22	2	1.39	0.045	0.045
PT9	30	4	3.62	0.300	**0.488**	22	2	2.00	0.409	0.426
PT35	30	3	2.89	0.467	0.453	22	2	1.87	0.182	0.169
PT6	30	5	3.52	0.467	0.563	22	3	2.39	0.500	0.475
PT12	30	4	3.60	0.567	0.610	22	3	2.87	0.545	0.506
PT25	29	10	6.10	0.586	0.727	22	5	3.77	0.591	0.691
PT4	29	6	4.50	0.897	0.753	21	6	4.79	0.857	0.688
PT24	30	3	2.99	0.200	**0.640**	22	3	2.39	0.364	0.489
Average	29.76	4.47	3.66	0.541	0.605	21.94	3.18	2.78	0.476	0.483
SE	0.136	0.529	0.303	0.047	0.036	0.059	0.312	0.256	0.060	0.052

Table 3.6 Allelic diversity and heterozygosity of black robin populations on Rangatira and Mangere islands, including a random subsample from the Rangatira population. n = number of individuals sampled, A = number of alleles per locus, AR = allelic richness per locus (in terms of rarefied number of alleles), H_O = observed heterozygosity, H_E = heterozygosity expected under Hardy-Weinberg equilibrium, SE = standard error. H_E values in bold represent significant deviation from HWE.

	Rangatira					**Rangatira subsample**					**Mangere**				
Locus	**n**	**A**	**AR**	**H_O**	**H_E**	**n**	**A**	**AR**	**H_O**	**H_E**	**n**	**A**	**AR**	**H_O**	**H_E**
PT37	115	2	1.92	0.261	0.253	29	2	1.98	0.345	0.334	30	1	1.00	-	-
PT18	114	2	2.00	0.360	0.417	30	2	2.00	0.367	0.413	29	2	2.00	0.379	0.470
PT2	115	3	2.09	0.348	0.330	30	2	1.98	0.400	0.325	28	2	2.00	0.357	0.416
PT26	113	3	1.20	0.027	0.026	30	1	1.00	-	-	30	2	2.00	0.667	0.472
PT7	114	3	2.88	0.614	0.604	30	3	2.69	0.500	0.539	30	3	2.92	0.633	0.597
PT10	115	2	2.00	0.417	0.470	30	2	2.00	0.533	0.472	27	2	2.00	0.370	0.492
PT39	115	2	2.00	0.357	0.435	30	2	2.00	0.433	0.440	30	2	1.44	0.067	0.066
PT27	115	3	2.22	0.417	**0.382**	30	3	2.43	0.367	0.396	27	2	1.91	0.259	0.230
PT40	113	2	2.00	0.504	0.495	30	2	2.00	0.533	0.506	30	2	2.00	0.533	0.452
PT1	114	5	3.22	0.693	0.657	30	4	3.21	0.633	0.632	30	4	3.36	0.767	0.640
PT38	115	2	1.93	0.226	0.265	30	2	1.99	0.300	0.345	30	2	2.00	0.467	0.506
PAU26	115	2	1.99	0.391	0.365	30	2	1.97	0.367	0.305	30	2	1.70	0.133	0.127
TG-02	114	2	2.00	0.404	0.413	30	2	2.00	0.300	0.440	30	2	1.94	0.233	0.259
PCA12	88	2	2.00	0.466	0.492	29	2	2.00	0.379	0.499	30	2	2.00	0.400	0.472
PGM1	98	4	2.14	0.429	0.382	29	2	1.99	0.345	0.373	30	2	1.84	0.200	0.183
Average	111.5	2.60	2.10	0.394	0.399	29.8	2.2	2.08	0.414	0.430	29.4	2.13	2.01	0.390	0.384
SE	2.012	0.235	0.116	0.040	0.039	0.107	0.175	0.120	0.026	0.025	0.270	0.165	0.140	0.055	0.048

Table 3.7 Direct comparison of diversity between the Chatham Island tomtit and the Chatham Island black robin, for eight loci that were polymorphic in both species, pooled across both island populations for each species. n = number of individuals sampled, A = total number of alleles per locus, AR = allelic richness, H_O = observed heterozygosity, H_E = heterozygosity expected under Hardy-Weinberg equilibrium, SE = standard error. H_E values in bold represent significant deviation from HWE.

	Tomtits					**Black robins**				
Locus	**n**	**A**	**AR**	**H_O**	**H_E**	**n**	**A**	**AR**	**H_O**	**H_E**
PT37	52	2	1.94	0.231	**0.414**	145	2	1.64	0.207	0.208
PT18	52	3	2.85	0.615	0.656	143	2	1.95	0.364	0.428
PT2	52	6	3.59	0.654	0.740	143	3	1.91	0.350	0.348
PT26	52	4	3.29	0.519	**0.681**	143	3	1.73	0.161	**0.247**
PT7	52	5	3.61	0.788	0.747	144	3	2.75	0.618	0.621
PT10	52	2	1.93	0.442	0.406	143	2	1.99	0.406	0.486
PT39	52	2	1.90	0.365	0.369	145	2	1.91	0.297	**0.385**
PT27	50	7	3.26	0.540	0.599	142	3	1.96	0.387	**0.356**
Average	51.8	3.88	2.80	0.519	0.576	118.5	2.50	1.98	0.349	0.385
SE	0.250	0.693	0.268	0.062	0.055	0.567	0.189	0.118	0.049	0.046

3.4.5 Genetic differentiation

Between the two species, there was a very high and significant level of differentiation at the eight shared loci ($F_{ST} = 0.490$, $F'_{ST} = 0.938$, $P \leq 0.001$). There was a moderate level of differentiation between the tomtit populations on Rangatira and Mangere, $F_{ST} = 0.102$, $F'_{ST} = 0.228$, $P \leq 0.001$ (17 loci). For the Rangatira and Mangere black robin populations differentiation was moderate and significant $F_{ST} = 0.121$, $F'_{ST} = 0.205$, $P \leq 0.001$ (15 loci), and supports the presence of two independent clusters as determined by cluster analysis. Comparison of the Mangere sample of black robins with a random subsample of thirty black robins on Rangatira showed a minimal effect of sample size when compared to that of the total sample of 115 individuals ($F_{ST} = 0.124$, $F'_{ST} = 0.205$, $P \leq 0.001$). There was no significant genetic differentiation between populations in Woolshed Bush and Top Bush on Rangatira Island ($F_{ST} = 0.003$, $P = 0.689$).

3.4.6 Identifying dispersers

Tests for the presence of dispersing individuals in STRUCTURE ver. 2.3.4 (Pritchard *et al.* 2000) identified one individual as a likely descendant of a disperser between island populations in the Mangere tomtit population (see Table 3.8). This individual possesses alleles rare in the Mangere population (for example, at locus PT5 this individual has allele 250 which is not otherwise found in the Mangere population sampled, but is common in the Rangatira population). The mean probability (± SE) of an individual being from the population it was sampled in was 0.956 ± 0.007 for black robins, and 0.936 ± 0.021 for tomtits. Four black robins were detected in the Rangatira black robin population as likely to have descended from dispersers (see Table 3.8). This was due to all four individuals having an allele (238) at locus PT26 that is only otherwise found in the Mangere population. Individual B109427 is the progeny of B98938, and shares this allele. The parents of B109665 were included in genotyping, but did not have this allele.

Table 3.8 Identification of potential descendants of dispersing individuals from STRUCTURE analysis. B = black robin individual, followed by individual band number, A = tomtit individual. Own = probability individual derived from the sampled population, M1 = probability of being a disperser, M2 = probability of being a first generation descendant of a disperser, M3 = probability of being a second generation descendant of a disperser. All probabilities were averaged across ten repeats.

Individual	**Own**	**M1**	**M2**	**M3**
B109427	0.330	0.036	0.289	0.346
B98938	0.317	0.093	0.281	0.309
B109665	0.297	0.041	0.341	0.322
B81403	0.388	0.01	0.262	0.340
A104705	0.208	0.151	0.308	0.209

3.5 Discussion

While the level of genetic diversity in terms of heterozygosity and number of alleles found in the Chatham Island black robin is lower than that of its sister-species, the Chatham Island tomtit, the level of microsatellite diversity in the black robin is higher than that reported in six

other New Zealand species of birds (see Table 3.9). Given the extreme population bottleneck experienced by black robins, this result is somewhat unexpected, but may be at least partially explained by the method used in this study to isolate microsatellite loci. By using Illumina sequencing I was able to assess a large number of candidate microsatellites for testing. This method allowed for the preferential selection of loci that were likely to be highly variable, more so than is possible with the traditional enriched library method, or 454 sequencing (Castoe *et al.* 2012). Thus it is likely that the relatively high level of variation in the black robin is in part due to the ability to preferentially select for highly variable loci. For example, the analyses of the mohua (*Mohoua ochrocephala*) and South Island robin (*Petroica australis*) solely used primers developed for other species (Boessenkool *et al.* 2007; Tracy & Jamieson 2011), which may partly account for the relatively low diversity found in these species. Studies of the remaining species exclusively used primers specific to the species of interest, except in the analysis of the kaka (*Nestor meridionalis*), which used a combination of species-specific and cross-species loci (Sainsbury *et al.* 2006).

Five of the New Zealand species exhibiting lower heterozygosity than the black robin have experienced range reductions or population fragmentation, and conservation management has included translocations of small numbers to areas within the historic range or predator-free islands (Andrews *et al.* 2013; Baker *et al.* 2010; Boessenkool *et al.* 2007; Grueber *et al.* 2008; Tracy & Jamieson 2011). The yellow-eyed penguin is the exception, but the South Island population assessed is believed to have been established from a small number of founders, which is likely to have carried limited genetic diversity, and the modern population is prone to severe population fluctuations (Boessenkool *et al.* 2010). The black robin has the lowest mean number of alleles reported for any New Zealand bird species except the takahe (*Poryphyrio hochstetteri*), which has experienced a strong bottleneck and exists as a highly fragmented population (Grueber *et al.* 2008), and the Chatham Island snipe (*Coenocorypha pusilla*) (Baker *et al.* 2010), which has recovered in size following substantial range contraction.

Table 3.9 Comparison of genetic diversity of tomtits and black robins with other New Zealand bird species, ranked by expected heterozygosity from lowest to highest. IUCN ranking: CR = critically endangered, E = endangered, V = vulnerable, LC = least concern. n = number of individuals sampled, loci = number of microsatellite loci used, A = mean number of alleles per locus, H_E = mean expected heterozygosity.

Species	Common name	IUCN ranking	n	Loci	A	H_E
Coenocorypha pusilla[1]	Chatham Snipe	V	21	9	1.44	0.034
Mohoua ochrocephala[2]	Yellowhead/Mohua	E	155	11	3.18	0.352
Cyanoramphus malherbi[3]	Orange-fronted kākāriki	CR	23	18	3.28	0.354
Petroica australis[4]	South Island robin	LC	516	10	2.81	0.365
Poryphyrio hochstetteri[5]	South Island takahē	E	25	19	2.32	0.379
Megadyptes antipodes[6]	Yellow-eyed penguin[a]	E	249	10	3.00	0.380
Petroica traversi	Chatham Island black robin	E	145	15	2.67	0.408
Nestor notabilis[7]	Kea	V	410	15	4.53	0.439
Nestor meridionalis[8]	Kaka	E	126	8	7.75	0.460
Himantopus novaezelandiae[9]	Black stilt/Kakī	CR	21	8	3.50	0.521
Petroica macrocephala chathamensis	Chatham Island tomtit	LC	52	17	3.82	0.544
Callaeas cinerea wilsoni[10]	North Island kōkako	E	49	4	5.75	0.556
Xenicus gilviventris[11]	South Island rock wren	V	134	14	6.50	0.564
Apteryx mantelli[12]	Northern brown kiwi	E	35	5	7.00	0.605
Anthornis melanura[13]	Bellbird	LC	315	8	5.43	0.621
Notiomystis cincta[14]	Stitchbird/Hihi	V	269	19	4.86	0.645

1. Baker *et al.* (2010), 2. Tracy and Jamieson (2011), 3. Andrews *et al.* (2013), 4. Boessenkool *et al.* (2007), 5. Grueber *et al.* (2008), 6. Boessenkool *et al.* (2010), 7. Dussex *et al.* (2015), 8. Sainsbury *et al.* (2006), 9. Hagen *et al.* (2011), 10. Hudson *et al.* (2000), 11. Weston and Robertson (2015), 12. Shepherd and Lambert (2006), 13. Baillie *et al.* (2014), 14. Brekke *et al.* (2011). a) South Island population.

The black robin also has low genetic diversity when compared to threatened passerine species around the world (see Table 3.10). Among those species with lower heterozygosities than the

black robin, the three species of mockingbirds (*Mimus* spp.), and the Nihoa millerbird (*Acrocephalus familiaris kingi*) are island endemic species (Addison & Diamond 2011; Hoeck *et al.* 2010), while the two remaining species have experienced range contraction and population fragmentation (Di Giacomo *et al.* 2015; Saranathan *et al.* 2007). Although the Chatham Island tomtit has an IUCN Red List ranking of Least Concern (BirdLife International 2016a), it has similar levels of diversity when compared to species that are deemed to be more vulnerable among New Zealand birds, and other passerine species.

The reduction in population size and extirpation from parts of its historic range have likely reduced genetic diversity of the Chatham Island tomtit, though to a lesser degree than in the black robin. These markers were developed for black robins, so the ascertainment bias should show a greater level of diversity at these loci in the black robin compared to the tomtit. However the results show that the strong impact of random genetic drift over the population history of the black robin has outweighed any effect of the ascertainment bias that would typically result in reduced diversity measured in non-target species. The lower variation in the smaller Mangere populations of both species clearly illustrates how the effects of drift are greater in small populations, resulting in greater loss of alleles and reduced heterozygosity than in the larger Rangatira populations.

Table 3.10 Comparison of genetic diversity of tomtits and black robins in this study with that of other passerine species from around the world, ranked from lowest to highest expected heterozygosity. These species were ranked as critically endangered, endangered, vulnerable, or near threatened in the IUCN Red List between 2011 and 2015. Of the 608 species achieving this ranking, studies estimating genetic diversity using microsatellite data were available for the 22 species listed here, and five additional passerines listed in Table 3.9. N = number of individuals sampled, Loci = number of loci used, nA = mean number of alleles (* = only mean allelic richness reported), H_E = mean expected heterozygosity, I = island endemic species, M = mainland species. IUCN status: CR = critically endangered, E = endangered, V = vulnerable, LC = least concern, NT = not threatened. a) Estimated number of mature individuals at the time of IUCN ranking.

Species	**Common name**	**Island or mainland**	**IUCN status**	**Estimated population size[a]**	**N**	**Loci**	**nA**	**H_E**
Mimus trifasciatus[1]	Floreana mockingbird	I	CR	50	117	16	1.72	0.167
Mimus macdonaldi[2]	Espanola mockingbird	I	V	600-1700	97	16	2.13	0.201
Acrocephalus familiaris kingi[3]	Nihoa millerbird	I	CR	650	139	3	2.00	0.364
Mimus melanotis[4]	San Cristobal mockingbird	I	E	5300	37	16	3.13	0.366
Procnias tricarunculata[5]	Three-wattled bellbird	M	V	6000-15000	44	7	6.00	0.380
Alectrurus risora[6]	Strange-tailed Tyrant	M	V	6000-15000	68	8	3.69	0.405
Petroica traversi	Chatham Island black robin	I	E	290	145	15	2.67	0.408
Telespiza cantans[7]	Laysan finch	I	V	1500-7000	166	9	2.56	0.412
Stipiturus mallee[8]	Mallee emu-wren	M	E	7500-35500	72	12	3.45*	0.470
Turdus lherminieri[9]	Forest thrush	I	V	2500-10000	331	10	3.27*	0.528
Petroica macrocephala chathamensis	Chatham Island tomtit	I	LC	2000	52	17	3.82	0.544
Atlapetes pallidiceps[10]	Pale-headed brushfinch	M	E	226	91	11	3.37*	0.560
Pseudonestor xanthophrys[11]	Maui parrotbill	I	CR	250-540	85-129	12	7.25	0.609

Table 3.10 continued

Species	Common name	Island or mainland	IUCN status	Estimated population size[a]	N	Loci	nA	H_E
Pomarea dimidiata[12]	Kakerori/Rarotonga monarch	I	V	310	81	7	4.00	0.630
Aphelocoma coerulescens[13]	Florida scrub-jay	M	V	4000-6000	1028	20	9.60	0.667
Agelaius xanthomus[14]	Yellow-shouldered blackbird	I	E	1250	63	9	6.22	0.680
Spizella wortheni[15]	Worthen's sparrow	M	E	100	100	12	6.00	0.691
Dendroica kirtlandii[16]	Kirtland's warbler	M	NT	2400	68	17	5.54*	0.692
Formicivora paludicola[17]	Sao Paulo Marsh antwren	M	-	<300	57	17	5.2	0.700
Oreomystis bairdi[18]	Kauai creeper	I	CR	150-610	11	8	4.5	0.736
Dendroica chrysoparia[19]	Golden-cheeked warbler	M	E	Insufficient data	109	9	7.9	0.751
Dasyornis brachypterus[20]	Eastern bristlebird	M	E	2550	105	6	8.25	0.762
Dendroica cerulea[21]	Cerulean warbler	M	V	Insufficient data	154	5	15.6	0.770
Ammodramus caudacutus[22]	Saltmarsh sparrow	M	V	30000	387	10	8.4*	0.798

1. Hoeck *et al.* (2010), 2. Hoeck *et al.* (2010), 3. Addison and Diamond (2011), 4. Hoeck *et al.* (2010), 5. Saranathan *et al.* (2007), 6. Di Giacomo *et al.* (2015), 7. Tarr *et al.* (1998), 8. Brown *et al.* (2013), 9. Arnoux *et al.* (2014), 10. Hartmann *et al.* (2014), 11. Mounce *et al.* (2015), 12. Chan *et al.* (2011), 13. Coulon *et al.* (2008), 14. Liu (2015), 15. Canales-Delgadillo *et al.* (2012), 16. Wilson *et al.* (2012), 17. de Camargo *et al.* (2015), 18. Eggert *et al.* (2009), 19. Lindsay *et al.* (2008), 20. Roberts *et al.* (2011), 21. Veit *et al.* (2005), 22. Walsh *et al.* (2012).

The presence of more than four alleles at one locus (PT1) in the black robin was unanticipated. At the time of the population bottleneck in 1980, there were five remaining individuals, only two of which were successful in raising offspring that survived and reproduced. The maximum possible number of alleles that can be passed on through a single-pair bottleneck in a diploid species is four, two from each parent. There are three possible scenarios that may explain the presence of more than four alleles. Firstly and most likely, is via mutation, as there were no other populations available to allow gene flow via dispersal. Mutation occurs at a slow rate, and may have been hindered by slow population growth in the recovering population. Secondly, there may have been unknown black robin individuals remaining on Mangere Island at the time of the translocation of the species from Little Mangere, and that may have bred with any of the five translocated individuals, and this could have resulted in more than four alleles being passed on in the population. However, this is extremely unlikely as no black robins were observed on Mangere at any time prior to the translocation, and no unbanded individuals were observed following the translocation of the species to Mangere. The third possibility is that there may have been a low level of extra-pair copulation occurring within the remnant population, where the sole breeding female, Old Blue, may have engaged in copulations with males other than her known partner. However, there was only one other male aside from the partner of Old Blue until the population began to grow. No evidence of extra-pair copulations have been recorded, but observational data typically under-estimates levels of extra-pair copulations in birds (Griffith *et al.* 2002). The potential likelihood of extra-pair paternity in the modern population is examined in Chapter Four.

Sample sizes of thirty individuals per population are thought to be sufficient to measure diversity within the populations (Hale *et al.* 2012). The results of the current study support this, with very similar levels of diversity between the full data set of 115 individuals on Rangatira compared to the random subsampling of thirty. As a larger proportion of the true population was sampled, a greater number of alleles was detected. However, there were minimal effects of sample size on measures of allelic richness (standardised for sample size) and expected heterozygosity, and on levels of differentiation between the two populations. While sampling of thirty individuals from each population is sufficient to accurately measure diversity and differentiation, the inclusion of additional samples that were later used to

investigate the likelihood of dispersal between islands and population substructuring on Rangatira did not alter the main conclusions.

The black robin population on Rangatira has been isolated for 26 years since establishment from a small subset of the Mangere population, which is derived from a single source population that survived for eighty years as a small population. In this study, I found that there is substantial differentiation between the two island populations. Similarly, substantial differentiation was found between tomtit populations on Rangatira and Mangere. This illustrates how strong genetic drift can lead to differentiation in a relatively short time, even between populations with relatively low levels of genetic diversity. Genetic drift results in different alleles being lost from each population at random, and the frequencies of the remaining alleles being altered at random. The Rangatira populations of both species are more resistant to loss of variation through drift, as the larger and more stable population sizes result in reduced allele fluctuations. Sufficient variation was still present in the black robin population to allow such strong and rapid differentiation. While both island populations of robin remain small and will experience ongoing genetic drift, the lower level of diversity on Mangere indicates drift continues to have a stronger effect on the Mangere population, resulting in lower diversity than the Rangatira population, even though the Rangatira population was founded from a subset of the Mangere population. This may indicate that the Mangere population is particularly vulnerable to extinction due to continued loss of diversity and inbreeding in this closed population.

Two recently published studies (Kennedy *et al.* 2014; Weiser *et al.* 2016) have treated the Rangatira population as containing two separate populations inhabiting different forested areas. I found there is a lack of evidence for differentiation between the two forest populations. Both cluster analysis and comparison of differentiation between individuals in these forest areas found no significant genetic differentiation between Woolshed and Top Bush. The suggestion was initially made due to the potential for the presence of skuas and an aversion of flying across open spaces to limit dispersal, in addition to noticeable differences in forest type and black robin demography in terms of breeding success and population growth rates (Kennedy 2009). The genetic data presented here, in combination with evidence from a study of dispersal (M. Massaro, personal communication), show that Skua Gully is not

a barrier to dispersal, indicating that although the Chatham Island black robin is reluctant to fly over open areas, it is able to disperse across this distance. Thus there is no need to distinguish between individuals inhabiting these forest patches for future analyses.

It was hypothesised that the tomtits may be able to use Pitt Island as a stepping stone allowing dispersal between all three islands, but the results here indicate a similar level of differentiation between the Mangere and Rangatira populations compared with that measured between the isolated black robin populations. The presence of only a single bird in the Mangere tomtit population that is identified as a potential descendant of a disperser indicates that while dispersal between the islands may be possible, it appears to have only occurred historically at a very low frequency. Potential future research including Pitt Island tomtit samples would be beneficial to compare levels of diversity and differentiation between all three populations across the species range. The larger population on Pitt Island is likely to have greater variation than the overall level found in this study and may help to clarify whether the Pitt Island population may have acted as a stepping stone for dispersal between Rangatira and Mangere.

The results of analysis of dispersal for the black robin populations revealed four individuals with mixed ancestry in the Rangatira population, but no true dispersers. This provides evidence that there is no naturally occurring dispersal between the two island populations, as expected given that robins avoid flying across open areas, and there is substantial differentiation between these populations. Any gene flow between the populations would prevent populations from differentiating, as alleles that may drift to a low frequency in one population could be restored through dispersal of individuals from the second population. The four individuals indicated as descendants of dispersing birds were identified as such because they all share an allele that is only found in these four individuals on Rangatira, while this allele is common within the Mangere population. This allele is most likely a remnant from the initial population establishment on Rangatira, occurring at low frequency in the modern population. The presence of such an allele within the population is indicative of the strength and random nature of genetic drift, such that in one population an allele has drifted to a high frequency, yet in the other population, it has drifted to an extremely low frequency.

The presence of distinct alleles that do not occur in the other black robin population (i.e., private alleles) indicates that sequential reciprocal translocations of individuals between islands may assist in reinforcing diversity within both populations. Individuals with low levels of relatedness should be preferentially selected for transfer. Such individuals are more likely to carry novel alleles at loci throughout the genome, including at loci associated with fitness, and so should improve average fitness as these alleles spread into the new population, and encourage population growth. These island habitats are similar and so it is unlikely that individuals have adaptive traits that are more advantageous on one island than the other.

However, the population on Mangere appears to be at carrying capacity (Kennedy 2009). This indicates the benefits of translocating individuals to Mangere may be limited, as insufficient habitat may limit population growth and the effective spread of new variation through the population. Therefore, in addition to reciprocal translocations, the establishment of a third population is recommended, using individuals sourced from both Rangatira and Mangere, and for repeated translocation events to maximise genetic variation in the new population. This third population would act as further insurance against catastrophic events, allow significant population growth beyond what is currently possible, and reduce extinction risk of the species. The larger combined source populations would allow a substantial number of individuals to be used for establishment while minimising risks to both existing populations. To further minimise such risks, care must be taken when selecting individuals for translocation. Selection of unpaired juveniles that can form new pair bonds with individuals from the other island should minimise the effects on the existing islands, while paired adults with proven breeding success should be included to ensure that the population can increase. Further analysis and modelling will be required to determine sufficient numbers to establish a self-sustaining population, and the size and frequency of sequential translocations. A recent study using a new model, AlleleRetain, suggested that forty black robin individuals would be sufficient to establish a self-sustaining population, based on simulations of allelic diversity likely to be present in the post-bottleneck population (Weiser 2014). It will now be possible to conduct modelling that includes the true levels of allelic diversity in the current populations based on the findings here, to allow more accurate estimations of founder size to be made. In the period following initial translocation, intensive monitoring will be required to track population numbers, sex-ratios, numbers of breeding pairs, and breeding success and recruitment, to comprehensively evaluate the success of

establishment, and to minimise the effects of disease, parasites, and storm events that may have led to the failure of the previous establishment attempt. Also the reduction in variation due to the founder effect may have less impact in the long term than loss of variation due to genetic drift, particularly on Mangere, if the populations remain as they are.

In regards to potential sites for population establishment, the 2001 - 2011 Black Robin Recovery Plan (DOC, 2001a) highlights the goal to return the species to Little Mangere Island, forty years after extirpation due to translocation of all remaining individuals to Mangere. Without significant habitat recovery on Little Mangere, the estimate of sufficient habitat for a maximum of 35 individuals (Kennedy 2009) remains. This would be an extremely small population, with the risk of increasing inbreeding and strong genetic drift reducing variation in this population without regular translocations of unrelated birds. If the hypothesis that the Mangere and Little Mangere populations may have historically acted as a single population is true, with dispersal deemed possible across the 260 m distance between habitats (Kennedy 2009), then such dispersal may help to reduce inbreeding and drift in each population. If the population on Mangere is limited by slow habitat regeneration, dispersal of individuals to Little Mangere may be currently occurring. However, the difficulty of accessing Little Mangere, which in part resulted in the decision to translocate the population initially to Mangere Island (Butler & Merton 1992), has prevented any investigation of whether birds have naturally dispersed to Little Mangere.

The potential for establishment of a third self-sustaining population on Pitt Island was discussed in the 2001 - 2011 Black Robin Recovery Plan (DOC, 2001a), but is dependent on the removal of invasive predators, most notably cats (D. Houston, personal communication). Pitt Island (6325 ha) has a large area of established forest (Caravan Bush) into which the population could establish, and the Ellen Elizabeth Preece Reserve remains a viable location for establishment. The established forest on Pitt Island may be less susceptible to storm damage that frequently has negative impacts on population size on Rangatira Island (D. Houston, personal communication). Establishment of a third population would be best conducted in tandem with reciprocal translocation between islands. Ultimately, population establishment would reduce the extinction risk of the Chatham Island black robin, as it would be more resilient in both the short-term where it will be less vulnerable to population

fluctuations and stochastic events, and the long-term where maintaining diversity will ensure greater evolutionary potential to allow the species to adapt to future change. In addition, once translocations cease, isolation may result in a similar pattern of differentiation occurring between populations, particularly in the short term when the new population is still relatively small.

In summary, the Chatham Island black robin has low genetic diversity due to its history of existing as a small population, leading eventually to the extreme bottleneck event in 1980. The two island populations have differentiated from one another over 26 years due to strong genetic drift acting separately since isolation. No population structuring was found on Rangatira Island. The best course of action to conserve the remaining genetic diversity and maximise evolutionary potential would be to establish a third population of Chatham Island black robins on Pitt Island, allowing for substantial population growth. At the minimum, reciprocal translocations of birds should be carried out between the two island populations to reinforce the standing level of genetic diversity, and reduce the extinction risk of this endangered species.

Chapter Four

Detection of extra-pair paternity in a socially monogamous endangered passerine

4.1 Abstract

The evolution of extra-pair copulations resulting in extra-pair paternity across a wide array of bird species is hypothesised to have arisen due to direct or indirect benefits acquired by females engaging in such a strategy. Genetic monogamy is much rarer than observational data would suggest among bird species, with an average of 11% of all offspring estimated to be the result of extra-pair copulations. Direct or indirect benefits of extra-pair matings to females include access to resources, or maximising the genetic diversity of offspring, thereby improving female fitness. Among small island populations, loss of genetic diversity through genetic drift may reduce such indirect benefits, but extra-pair paternity may allow the avoidance of inbreeding in these small populations. The socially monogamous Chatham Island black robin *Petroica traversi* is limited to populations on two small islands, with a total species size of only 290 individuals. Microsatellite genotyping detected 8.2% of offspring to have resulted from extra-pair paternity. There was no difference in relatedness between social pairs that produced extra-pair offspring and those that produced only within-pair offspring. Heterozygosity of extra-pair offspring was not greater than for within-pair offspring. While the underlying reason for the evolution of extra-pair copulations as a strategy by females in this species is unknown, the detection of extra-pair paternity has implications for conservation management. The occurrence of extra-pair paternity reduces confidence in the known pedigree based on observational data, and therefore it is recommended that management decisions should use molecular methods to infer relatedness, rather than rely solely on the known pedigree.

4.2 Introduction

Offspring resulting from copulations outside of the social pair are regarded to have extra-pair parentage. Extra-pair paternity (EPP) is found in around 90% of all passerine species (Griffith *et al.* 2002). Approximately 11% of offspring are the result of extra-pair copulations (EPCs)

by females in bird species that appear socially monogamous (Griffith *et al.* 2002). Variation in rates of EPP between and even within species can be high, and the reasons underlying such variation are complex (Petrie & Kempenaers 1998). Extra-pair males gain direct fitness benefits from EPCs, as they can increase their reproductive output at low cost, whereas females are limited by the number of eggs they can produce per season, and so cannot easily increase their reproductive output (Westneat *et al.* 1990). However, they can improve the quality of their offspring. The frequency of EPP in a population may be determined from a combination of benefits to females, or other ecological factors. The benefits of seeking EPCs by females may include direct benefits such as access to additional resources, or indirect benefits including improved fertility, increased genetic diversity or beneficial genes in offspring, and improved genetic compatibility with the extra-pair partner (Eliassen & Kokko 2008; Foerster *et al.* 2003; Griffith *et al.* 2002).

If females seek EPCs to improve the genetic quality of their offspring, the rate of EPP in a population may be associated with the amount of variation in male quality, and consequently with the level of genetic variation in the population (Petrie & Lipsitch 1994). When there is a high level of variation in quality between males, females may seek EPCs with better quality males more frequently than in populations with relatively little variation in male quality. Therefore, if improving offspring quality is the key driver for females to seek EPCs, populations with low genetic diversity would be expected to have low rates of EPP compared to similar populations with relatively high diversity (Petrie & Lipsitch 1994). However, small island populations typically have reduced genetic diversity compared to similar mainland populations (Frankham 1997), thereby limiting the benefit of using EPCs to maximise genetic diversity of offspring (Griffith *et al.* 2002). Moreover, variation in sexually selected characteristics, such as plumage colouration, may be reduced in island populations compared to mainland populations (Doutrelant *et al.* 2016; Grant 1965). While the low levels of genetic variation in such populations may predict low levels of EPP, high breeding densities or breeding synchrony may instead drive the frequency of EPP (Griffith *et al.* 2002; Krokene & Lifjeld 2000; Westneat & Stewart 2003).

Furthermore, EPCs by females may have evolved as a strategy of inbreeding avoidance (Griffith *et al.* 2002; Kempenaers *et al.* 1999; Tregenza & Wedell 2000). Inbreeding

increases homozygosity of the progeny of matings between close relatives, resulting in the increased expression of deleterious recessive alleles (Keller & Waller 2002). This process, known as inbreeding depression (Keller & Waller 2002), has negative effects on fitness of inbred offspring at a range of life stages, including reduced fertility, hatching success, and longevity in birds (Jamieson *et al.* 2006). If there is a high level of inbreeding in the local population, females may seek EPCs with unrelated males to improve genetic diversity of offspring, and so avoid the negative consequences of inbreeding (Szulkin *et al.* 2013). A meta-analysis of 33 bird species found that higher relatedness between breeding pairs led to an increased occurrence of EPP (Arct *et al.* 2015), indicating that EPCs can be used as a strategy of inbreeding avoidance. Conversely, in highly outbred populations, females may seek EPCs with more closely related males, or geographically nearby males, who are more likely to share traits that are beneficial to the local environment, and so can ensure inheritance of adaptive traits by progeny (Foerster *et al.* 2003).

This study assesses the frequency of EPP in a population of the Chatham Island black robin (*Petroica traversi*), a sexually monomorphic, socially monogamous member of the Petroicidae family of Australo-Papuan robins (Miller & Lambert 2006). A rate of EPP (calculated as the number of pairs which produced EPP offspring divided by the total number of pairs assessed) of 1.9% was detected in the closest congener with data available, the New Zealand robin (*Petroica australis*), from microsatellite genotyping of 54 social pairs and 198 offspring (Taylor *et al.* 2008). Extensive observational data of the Chatham Island black robin has been collected over the past 35 years, with no observations of EPCs ever recorded (Butler & Merton 1992; Kennedy 2009). The long history of small population size in black robins culminated in an extreme population bottleneck in 1980, following which all individuals alive today are descended from a single breeding pair (Butler & Merton 1992). This has resulted in a relatively low level of genetic diversity (see Chapter Three) and one of the highest rates of inbreeding in a wild bird population (Ardern & Lambert 1997; Massaro *et al.* 2013a), with substantial inbreeding depression in terms of fledgling and juvenile survival (Kennedy *et al.* 2014; Weiser *et al.* 2016). These studies calculated inbreeding coefficients from pedigree data based on behavioural observations of individuals on Rangatira Island, and a very low level of EPP was assumed ($\leq 2\%$), based on that found in the New Zealand robin (Kennedy *et al.* 2014; Taylor *et al.* 2008; Weiser *et al.* 2016). With the polymorphic microsatellite loci developed for the black robin in Chapter Two, it is now possible to test

whether this assumption is correct. To improve the future viability of the species, conservation management may include translocations of birds between islands, or the founding of a third population, which may be less effective than expected if unrecognised EPP is occurring and birds are selected for translocations based on the known observational pedigree (Pemberton 2008).

In this chapter I assess 61 offspring across 35 family groups to investigate whether there is any evidence of extra-pair paternity in the Chatham Island black robin. If females are seeking EPCs to improve offspring quality, the low level of genetic variation in the population may predict a low rate of EPP. However, if EPCs are used as a strategy to avoid inbreeding, the rate of EPP may be relatively high compared to that of the New Zealand robin. I identify genotypic discrepancies between parents and offspring, and subsequently attempt parentage assignment using CERVUS ver. 3.0 (Kalinowski *et al.* 2007). I compare estimates of relatedness between social pairs to determine whether females that are more closely related to their social partner are more likely to produce EPP offspring than those in less related pairs, as may be predicted if this strategy has evolved as a mechanism to reduce inbreeding in the population (Szulkin *et al.* 2013). I also assess whether EPP offspring are more genetically diverse (in terms of individual heterozygosity) than offspring resulting from social monogamy.

4.3 Methods

4.3.1 Sampling and extraction

Black robin blood spot and feather samples were collected from 2008 – 2011, along with the location of breeding or natal sites of individuals in family groups (Garmin GPSMAP60CSx, < 10 m). DNA was extracted from the majority of samples for a previous study (Cubrinovska et al. 2015). For the current study, I extracted DNA from an additional 71 black robin individuals sampled in 2010 and 2011 following the blood spot protocol (Invitrogen PureLink Genomic DNA kit user manual). These individuals were genetically sexed and verified against observational data, with no discrepancies found. Sexing primers P2 and P8 (Griffiths *et al.* 1998) were used for genetic sexing, using the PCR protocol as described by Fridolfsson and Ellegren (1999). PCR products were run on a 3% agarose gel at 120 V for three hours.

Males display a single band of approximately 390 base pairs, while females have an additional band of 430 base pairs. All individuals that displayed a single band were amplified and visualised a second time to ensure no second band was present. Two known males and two known females were used as controls, and were always included in thermocycling reactions and when running gels.

4.3.2 Individuals selected for genotyping

To determine rates of EPP in the black robin, family groups on Rangatira Island consisting of parents and all known offspring across years with samples available were selected for parentage analysis, totalling 127 individuals comprising 35 family groups, including 61 offspring. In addition, the remaining 36 available adult male samples and 13 female samples collected from 2008 to 2010 and not included in any of the family groups were genotyped for inclusion in parentage analysis once mismatches within family sets were detected. In total, 176 Rangatira Island black robin individuals were genotyped. All family groups were compared against the original data sheets by a second person to ensure correct identification of individuals comprising family groups.

4.3.3 Microsatellite loci

All 15 loci that were polymorphic in black robins described in Chapter Three were used to genotype birds in this study, using the amplification and scoring protocols described. Negative controls, consisting of the reaction mix with no DNA added, were incorporated in every PCR amplification and subsequent genotyping to minimise genotyping error and test for contamination. Genotyping error was quantified by amplifying, genotyping, and scoring 14% of all samples genotyped for the black robin study twice for each locus. The error rate was calculated by dividing the number of errors by the total number of samples repeated (Hoffman & Amos 2005). Estimation of the genotyping error rate is a necessary parameter for parentage analysis, as genotyping error can increase the Type II error rate, reducing statistical power to assign putative parents or exclude observed parents (Hoffman & Amos 2005).

To determine power of parentage analysis for this population, mean polymorphic information content (PIC; a measure of the usefulness of the markers for linkage analysis) and total non-exclusion probabilities (the likelihood that an unrelated individual cannot be excluded as a parent or sibling) for combined loci were calculated in CERVUS ver. 3.0 (Kalinowski *et al.* 2007) based on allele frequencies at 15 loci for the 115 individuals selected for the analyses of diversity and differentiation described in Chapter Three. This data set was used as it excluded known relatives, and so limits bias of allele frequencies that may be caused by the inclusion of close relatives. To compare the power of paternity analysis with that of a more genetically diverse population, mean PIC and combined exclusion probabilities of this data set were compared with those derived from a set of thirty tomtits (*Petroica macrocephala chathamensis*) from Rangatira Island at the eight loci polymorphic in both species, as described in Chapter Three.

All individual genotypes (n = 176) were tested for genotype matches using GENALEX ver. 6.5 (Peakall & Smouse 2006; Peakall & Smouse 2012), and the proportion of pairs with identical genotypes was compared to the probability of non-exclusion of identity calculated with CERVUS.

4.3.4 Identification of individuals resulting from extra-pair paternity

Genotype data for all family groups was assessed to detect mismatches in genotypes between social parents and offspring. When a female engages in EPCs, she is the mother of offspring in her nest, but the social partner of this female would not be the biological father of the offspring. When a male engages in EPCs, he is most likely the father of offspring in his nest, and his social partner is the biological mother of these offspring, but he is also the father of offspring in nests of other social pairs. Therefore, the social mother was assumed to be the true mother of offspring in the nest. Thus, one allele at each locus in the offspring genotype was assumed to be derived from the social mother (unless no alleles at a locus in the offspring could have come from the social mother), with the second allele compared against the paternal genotype. Offspring were allocated to one of five groups (see Figure 4.1).

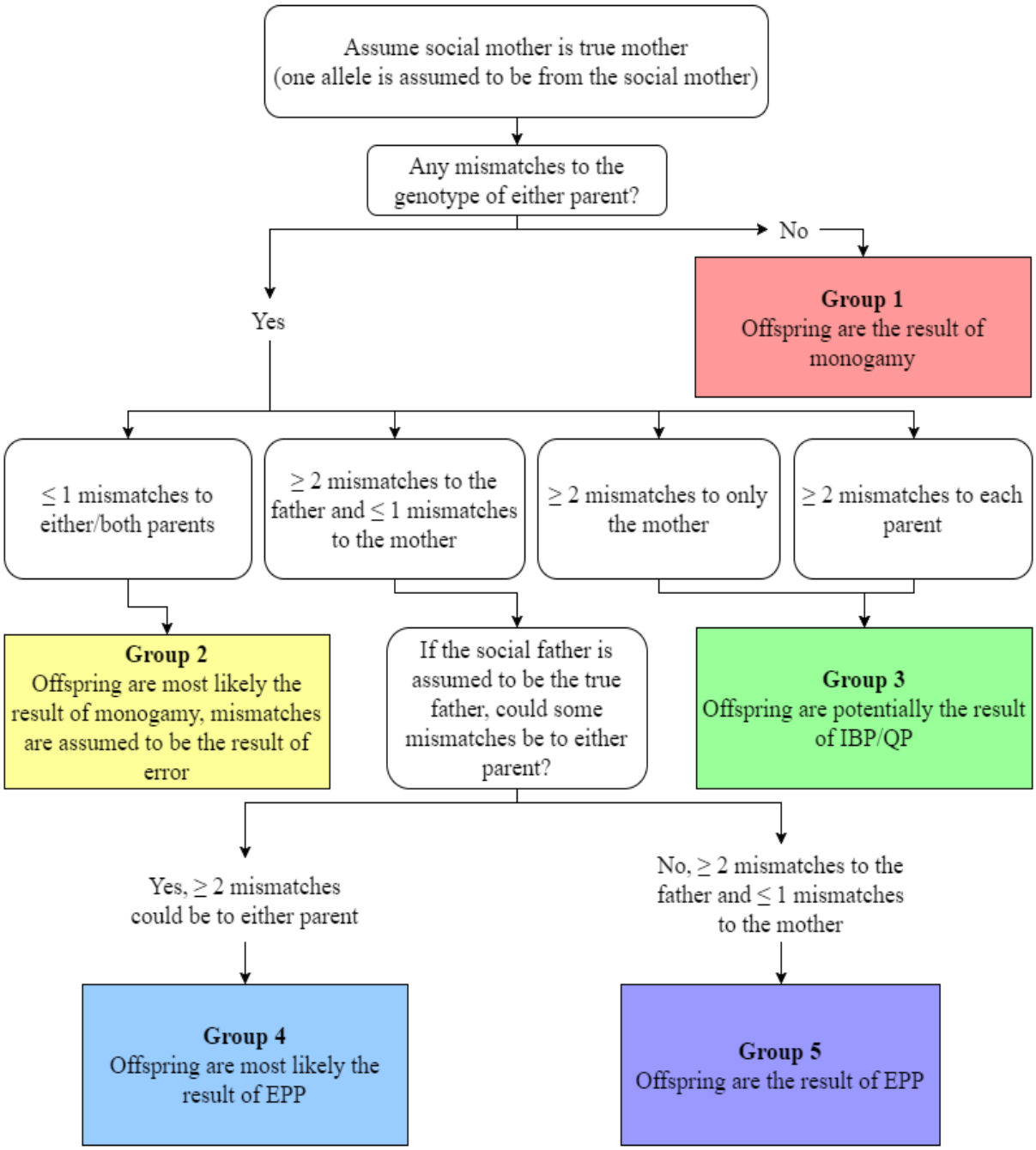

Figure 4.1 The process used to determine whether offspring were the result of extra-pair paternity. EPP = Extra-pair paternity, IBP = Intra-specific brood parasitism, QP = Quasi-parasitism.

Offspring with no mismatches to either parent (Group 1) were assumed to be the result of social and genetic monogamy. Allelic mismatches to either parent at a single locus were discounted to prevent overestimation of the rate of EPP, and to take into account error due to potential null alleles or mutations (Marshall *et al.* 1998; Taylor *et al.* 2008). Offspring with

single locus mismatches were assumed to be the result of monogamy (Group 2). Offspring with mismatches at two or more loci to both social parents were identified as potentially the result of intraspecific brood parasitism or quasi-parasitism (Group 3). Intraspecific brood parasitism may occur if a female lays eggs in the nest of another pair. Quasi-parasitism is a form of intraspecific brood parasitism whereby the male breeds with an extra-pair female, and then encourages the extra-pair female to lay eggs in the nest of the social pair (Griffith *et al.* 2004). Offspring with multiple mismatches that could not clearly be assigned to one parent (and so could be mismatches to either parent) were assumed to be mismatched to the social father, and so were regarded as most likely the result of EPP (Group 4). Offspring were considered to be definitely the result of EPP if genotyping revealed allelic mismatches to only the social father at two or more loci (Group 5). Where multiple mismatches were detected, mismatched loci in offspring and social parents were re-amplified and re-genotyped. The EPP rate was calculated by dividing the number of pairs that produced at least one extra-pair young (Group 5 offspring) by the total number of pairs (35), as in Taylor *et al.* (2008). Although Group 4 offspring are assumed to be the result of EPP, they were excluded from this calculation to prevent overestimation of the rate of EPP.

4.3.5 Parentage analysis

Parentage analysis was conducted for all offspring using CERVUS ver. 3.0 (Kalinowski *et al.* 2007) to attempt to identify the most likely genetic fathers of EPP individuals (Group 4 and 5). Based on allele frequencies derived from the set of 115 Rangatira black robins calculated in CERVUS, parentage was assigned using the natural logarithm of the likelihood ratio (LOD score). The LOD score is calculated as the probability that a candidate parent is the true genetic parent of an individual, divided by the probability of that candidate parent not being the true parent, multiplied over all loci (Kalinowski *et al.* 2007; Marshall *et al.* 1998). A positive LOD score indicates the candidate parent is more likely to be the true parent than not the true parent. The delta value (Δ) is the difference between the LOD scores of the two most likely candidate parents, or when there is only a single candidate with a positive LOD score, Δ is the same as the LOD score. By comparing the distribution of simulated Δ values for correct assignments and false assignments (the likelihood of a parent other than the true genetic parents being identified as the most likely parent), the simulations produce a critical Δ value for the given level of confidence (Jones *et al.* 2010; Kalinowski *et al.* 2007; Walling *et*

al. 2010). Simulations of parentage analysis were performed over 10,000 randomisations to determine critical Δ values for confidence of assignment. CERVUS takes into account both the genotyping error rate, and the likelihood that not all parents have been sampled (Kalinowski *et al.* 2007). The list of candidate parents for each individual excluded known siblings or half-siblings born in the same year as the individual of interest, but included all other individuals genotyped, regardless of age or nesting location. To assess the effect of the estimated number of candidate parents per individual, simulations were compared with three, five, and ten candidate parents of each sex (approximately the breeding density, estimated at 2.53 breeding pairs per hectare (Kennedy 2009), double the breeding density, and more than three times the breeding density), and for the maximum number of candidate parents listed for each individual (85 candidate fathers and 61 candidate mothers). Larger numbers of candidate parents and lower proportions of candidate parents sampled are expected to reduce the power of analysis (Jones *et al.* 2010; Marshall *et al.* 1998). While breeding densities may provide a good indication of the number of individuals in the local neighbourhood, dispersal between breeding seasons has been observed of up to 732 m by females and 690 m by males (M. Massaro, personal communication), substantially increasing the number of candidate parents that may be in the vicinity during the breeding period. Therefore in the final analysis, the total number of candidate parents genotyped was used. I estimated the proportion of candidate females sampled as 65%, and the proportion of candidate males sampled as 83%, based on the 2011 population census of 94 adult females and 102 adult males on Rangatira Island (M. Massaro, personal communication). The population in 2010 numbered 186 adults (Massaro *et al.* 2013b), and so these estimates of the proportions of adults sampled are conservative, as they may underestimate the true proportion sampled. Parentage assignments were made with CERVUS default values of either a strict confidence level of 95%, or a relaxed confidence level of 80% to the individual with the highest LOD score. A candidate father within close proximity of the natal site is more likely to be the true father than a candidate located more distantly, and so linear distances between the natal site of offspring and the nesting site of the most likely candidate father were determined in GENALEX ver. 6.5 (Peakall & Smouse 2006; Peakall & Smouse 2012) based on GPS data (Garmin GPSMAP60CSx, < 10 m) collected during banding and sampling.

4.3.6 Heterozygosity, relatedness, and extra-pair paternity

If females seek EPCs to limit inbreeding, the heterozygosity of females may affect whether females seek EPCs (Foerster *et al.* 2003). To investigate whether females with extra-pair offspring were more heterozygous than genetically monogamous females, individual standardised heterozygosities were calculated using the formula described by Coltman *et al.* (1999):

$$HS = \frac{proportion\ of\ heterozygous\ loci\ typed}{mean\ heterozygosity\ of\ typed\ loci}$$

Heterozygosity of females producing EPP offspring (Group 4 and 5 offspring) were compared to those of females producing offspring that contained no allelic mismatches (Group 1 offspring). Differences in mean heterozygosities were tested for significance using the non-parametric Mann-Whitney U test. If females are seeking EPCs to avoid inbreeding or to improve genetic diversity of offspring, heterozygosity of EPP offspring (Group 4 and 5 offspring) may be expected to be greater than that of offspring produced through monogamy (Group 1 offspring), and so significance of these comparisons was also assessed using non-parametric Mann-Whitney U tests.

Queller and Goodnight's (1989) coefficient of relatedness (r_{xy}) was used to infer familial relationships between breeding pairs, between siblings, and between offspring and social fathers, using IDENTIX ver. 1.1 (Belkhir *et al.* 2002). r_{xy} ranges from -1 to 1, with -1 indicating outbred individuals, 0 for unrelated individuals, 0.25 for second-order relationships (i.e., half-siblings), and 0.5 for first order relationships (i.e., parent-offspring or full-siblings) (Blouin 2003). Relatedness values calculated between siblings, and between offspring and social fathers was used to confirm the identification of mismatched offspring as the result of EPP. Between-pair relatedness was assessed to determine whether females engaged in EPP when they were more closely related to their social partner, and so may support the hypothesis of EPP as a strategy to avoid inbreeding. Mean relatedness between social pairs with EPP offspring (Group 4 and 5 offspring) was compared to that of social pairs producing offspring with no allelic mismatches (Group 1 offspring) using Mann-Whitney U tests.

4.4 Results

4.4.1 Genotyping

Multilocus genotypes were obtained for 176 black robin individuals. Among the 35 family sets included for analysis, three adults were part of up to three different breeding pairs recorded in different years that were also included. Samples available for chicks per pair ranged from one to five (mean ± SE = 1.74 ± 0.171), with offspring samples available from up to three breeding seasons for a single pair. Genotyping error rates calculated from repeat genotyping described in Chapter Three was 1.42%. This error rate would predict a total of 37.5 errors across the 176 individuals genotyped at 15 loci included in parentage analysis.

4.4.2 Microsatellite loci

Both CERVUS (Kalinowski *et al.* 2007) and MICROCHECKER ver. 2.2.3 (Van Oosterhout *et al.* 2004) analyses detected potential null alleles at locus PT39 (null allele frequency estimates of 0.115 and 0.092 from CERVUS and MICROCHECKER respectively). However, PT39 was included in the final analyses, as it improved the power of the analysis, which was relatively low (from 0.739 up to 0.763 when PT39 is included). CERVUS is designed to incorporate a low frequency of null alleles in the error rate for parentage analysis, and the inclusion of PT39 did not alter whether social fathers were excluded as most likely true fathers, or identification of most likely true fathers in test simulations. Furthermore, potential null alleles at locus PT39 did not have any effect on identifying extra-pair offspring, as only two parent-offspring allelic mismatches were detected at this locus, and both these offspring had allelic mismatches across at least four other loci.

The results of CERVUS analysis of allele frequencies for the data set used to determine allele frequencies and observed and expected heterozygosities for the Rangatira robin population in Chapter Three (n = 115) found a polymorphic information content (PIC) ranging from 0.026 to 0.583 across the 15 loci (see Table 4.1). The combined probability that an unrelated parent could not be excluded when only the offspring genotype was known was 0.237, the combined probability that an unrelated male could not be excluded when both the offspring and maternal genotypes were known was 0.054, and the combined probability that an unrelated parent pair could not be excluded given the offspring genotype was 0.008 (see Table 4.1).

Mean PIC and combined exclusionary power was low in comparison to that of the sample of thirty tomtit individuals from Rangatira Island (see Table 4.2), and to other such studies of parentage (e.g. Taylor *et al.* (2008); Winternitz *et al.* (2015)).

Table 4.1 Parentage analysis parameters of 15 microsatellite loci in black robins. n = number of individuals genotyped, PIC = polymorphic information content, NE-1P = average non-exclusion probability for one candidate parent, NE-2P = average non-exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex, NE-PP = average non-exclusion probability for a candidate parent-pair, NE-I = average non-exclusion probability for identity of two unrelated individuals, NE-SI = average non-exclusion probability for identity of two siblings.

Locus	**n**	**PIC**	**NE-1P**	**NE-2P**	**NE-PP**	**NE-I**	**NE-SI**
PT37	115	0.220	0.968	0.890	0.815	0.591	0.772
PT18	114	0.329	0.914	0.836	0.747	0.428	0.650
PT2	115	0.280	0.946	0.857	0.767	0.499	0.711
PT26	113	0.026	1.000	0.987	0.974	0.948	0.974
PT7	114	0.517	0.819	0.691	0.548	0.243	0.510
PT10	115	0.359	0.890	0.821	0.729	0.392	0.614
PT39	115	0.339	0.906	0.830	0.740	0.415	0.637
PT27	115	0.320	0.927	0.832	0.733	0.444	0.671
PT40	113	0.371	0.879	0.814	0.721	0.379	0.599
PT1	114	0.583	0.780	0.630	0.474	0.191	0.471
PT38	115	0.229	0.965	0.885	0.809	0.576	0.762
PAU26	115	0.298	0.934	0.851	0.765	0.471	0.686
TG02-088	114	0.327	0.915	0.837	0.748	0.431	0.652
PCA12	88	0.369	0.880	0.815	0.722	0.381	0.601
PGM1	98	0.315	0.928	0.838	0.743	0.449	0.672
Combined probability of non-exclusion			0.237	0.054	0.008	<0.001	0.002

Table 4.2 Comparison of polymorphic information content (PIC) and non-exclusion probabilities for eight polymorphic loci in the Chatham Island black robin and the Chatham Island tomtit.

	Black robins	**Tomtits**
Number of individuals sampled	115	30
Mean PIC	0.299	0.521
Combined non-exclusion probability (1P)	0.513	0.129
Combined non-exclusion probability (2P)	0.246	0.030
Combined non-exclusion probability (PP)	0.098	0.003
Combined non-exclusion probability (I)	0.002	< 0.0001
Combined non-exclusion probability (SI)	0.046	0.005

Four pairs of individuals were found to contain identical genotypes (see Table 4.3). Of these four pairs, one was found to be a parent-offspring pair. There were no recorded familial relationships between members of the other three pairs.

Table 4.3 Pairs of individuals with identical genotypes from genotyping 176 black robin individuals. IDs are individual band numbers recorded for identification. Any loci with missing data recorded due to poor amplification are listed, and family relationships are recorded where known.

Identical pair IDs	**Missing data**	**Relationship**
B81404	PT1, PCA12	Unknown
B98999	PCA12	
B109648	PAU26	Unknown
B81170	-	
B109416	-	Parent - offspring
B109645	-	
B98835	-	Unknown
B98931	-	

4.4.3 Identification of individuals resulting from extra-pair paternity

I detected 21 offspring of 14 parent-pairs containing at least one allele that could not be derived from the recorded parent-pair, with between one and three offspring per pair containing allelic mismatches at between one and six loci (see Table 4.4). At least one mismatch occurred in each locus across all family groups sampled. The total number of mismatched loci detected between parents and offspring was 53 (mean number (± SE) of mismatches across all progeny was 0.88 ± 0.189). Following the detection of mismatches, 34 individuals including offspring and parents were re-amplified and re-genotyped at between one and five mismatched loci. Only a single scoring error was detected during this process. Of the 21 individuals containing mismatches to the social parents, six individuals contained mismatches at only a single locus (Group 2 offspring) and so were excluded from further analysis as these mismatches were assumed to be the result of error. As most offspring genotypes matched the social parents exactly, or contained only a single locus mismatch that could be due to error, it was assumed that the majority of the social parents were the true genetic parents.

When the social mother was assumed to be the genetic mother, 12 individuals across nine families contained allelic mismatches at two or more loci with the social father and fewer than two mismatches to the assumed mother, and so were assumed to be the result of EPP (Group 4 and 5 offspring; see Table 4.4). However, while it is more likely that such mismatches are the result of EPP than due to intraspecific brood parasitism, all mismatches in four of these individuals (B98997, B109635, B109401, and B109665) could be to either parent. Additionally, one individual (B109639) contained five mismatches to either parent and one mismatch to both, and individuals B98998 and B109633 contained two and four mismatches respectively to the social father when the social mother was assumed to be the true mother, but some of these mismatches could be to either or both parents. Therefore, while these seven individuals are assumed to be the result of EPP (Group 4 offspring), and grouped with Group 5 offspring for the purpose of further analysis, they are excluded from the calculation of the rate of EPP to provide a more conservative estimate. Only five offspring are identified as Group 5 offspring. Thus the rate of EPP (the number of family groups containing at least one Group 5 offspring divided by the total number of family groups assessed) was 14.3 %, with 8.2 % of all offspring found to be the result of EPP.

Inclusion of all 12 individuals categorised as Group 4 and 5 offspring would produce an EPP rate of 25.7%, with 19.7% of offspring assumed to be the result of EPP. One individual (B109648) contained multiple mismatches to both social parents, and one individual B109649 contained one mismatch to both parents and one mismatch that could be to either social parent. A single individual (B98982) was found to contain mismatches to the social mother at three loci. These three individuals may be the result of intra-specific brood parasitism or quasi-parasitism and so were identified as Group 3 offspring.

Table 4.4 Offspring containing mismatching genotypes to the social parents, categorised according to Figure 4.1. Individuals and social parents are identified using band numbers. Genotypes are listed as the number of base pairs for each allele at a locus. Offspring are grouped as in Figure 4.1: yellow = Group 2: mismatches assumed to be due to error, individuals most likely the result of monogamy, green = Group 3: offspring are potentially the result of intraspecific brood parasitism or quasi-parasitism, light blue = Group 4: offspring are most likely the result of EPP, but not included in the calculation of the rate of EPP, dark blue = Group 5: offspring are definitely the result of EPP.

Offspring	**Mother**	**Father**	**Locus**	**Offspring genotype**	**Maternal genotype**	**Paternal genotype**
B109406	B98948	B98801	PGM1	294/302	294/294	294/294
B109420	B81184	B98820	PT27	233/233	224/233	224/224
B109421	B81184	B98820	PAU26	193/195	195/195	195/195
B109434	B98829	B98955	PT10	326/338	326/326	326/326
B109655	B98960	B98870	PT1	347/360	360/360	322/343
B109664	B98961	B98862	PT2	202/220	220/220	220/220

Table 4.4 continued.

Offspring	Mother	Father	Locus	Offspring genotype	Maternal genotype	Paternal genotype
B109648	B81458	B81403	PT18	220/229	229/229	229/229
			PT39	107/107	109/109	107/107
			PT40	143/143	132/143	132/132
			PT1	343/343	343/360	322/360
			PGM1	294/294	302/302	294/302
B109649	B81458	B81403	PT18	220/220	229/229	229/229
			PT7	272/275	265/275	265/275
B98982	B98978	B98879	PT1	360/360	319/322	322/360
			TG02-088	262/262	261/261	262/262
			PCA12	100/100	108/108	100/100
B98997	B98949	B81442	PT1	343/360	322/343	343/343
			PAU26	193/195	195/195	195/195
B98998	B98949	B81442	PT18	220/220	220/229	229/229
			PT1	343/360	322/343	343/343
			PT38	101/101	101/105	105/105
			PAU26	193/195	195/195	195/195
B109401	B98872	B98868	PT2	202/220	202/202	202/202
			PT27	224/233	233/233	233/233
B109633	B81184	B98820	PT37	137/141	137/137	137/137
			PT7	265/265	265/275	272/275

Table 4.4 continued.

Offspring	Mother	Father	Locus	Offspring genotype	Maternal genotype	Paternal genotype
B109635	B98913	B109448	PT7	265/272	272/275	272/272
			PT1	322/343	343/343	343/360
			TG02-088	261/262	262/262	262/262
B109639	B98935	B98968	PT18	220/229	229/229	229/229
			PT7	265/265	275/275	275/275
			PT10	326/338	326/326	326/326
			PT39	107/109	107/107	107/107
			PCA12	100/108	108/108	108/108
			PGM1	294/302	294/294	294/294
B109665	B98936	B98871	PT26	208/238	208/208	208/208
			PT38	101/105	105/105	105/105
			PAU26	193/195	195/195	195/195
			PCA12	100/108	100/100	100/100
B98979	B98914	B98850	PT18	229/229	229/229	220/220
			PT27	233/233	233/233	224/236
			PT1	322/360	322/322	322/322
B109439	B81203	B81170	PT1	360/360	322/360	343/343
			PT38	101/105	105/105	105/105
			PGM1	302/302	294/302	294/294
B109634	B98913	B109448	PT7	275/275	272/275	272/272
			PT38	105/105	101/101	105/105
			PAU26	193/193	193/195	195/195

Table 4.4 continued.

Offspring	Mother	Father	Locus	Offspring genotype	Maternal genotype	Paternal genotype
B109640	B98948	B98801	PT18	229/229	229/229	220/220
			PT27	224/224	224/233	233/233
			PT40	143/143	143/143	132/132
B109661	B98949	B81442	PT1	322/360	322/343	343/343
			PCA12	100/100	100/100	108/108

4.4.4 Parentage analysis

Simulations of parentage analysis using CERVUS found that greater numbers of candidate parents per individual reduced the expected number of assignments of parentage (see Figure 4.2). This had a greater impact on assignments made with strict confidence (95%) than relaxed confidence (80%). Parentage assignment based on these simulations did not alter the number of parents with positive LOD scores per individual, or the identity of the most likely candidate parents, but did reduce the confidence in these assignments (see Figure 4.2). For the final analysis, the maximum number of candidate parents for a single individual was used, (see Table 4.5).

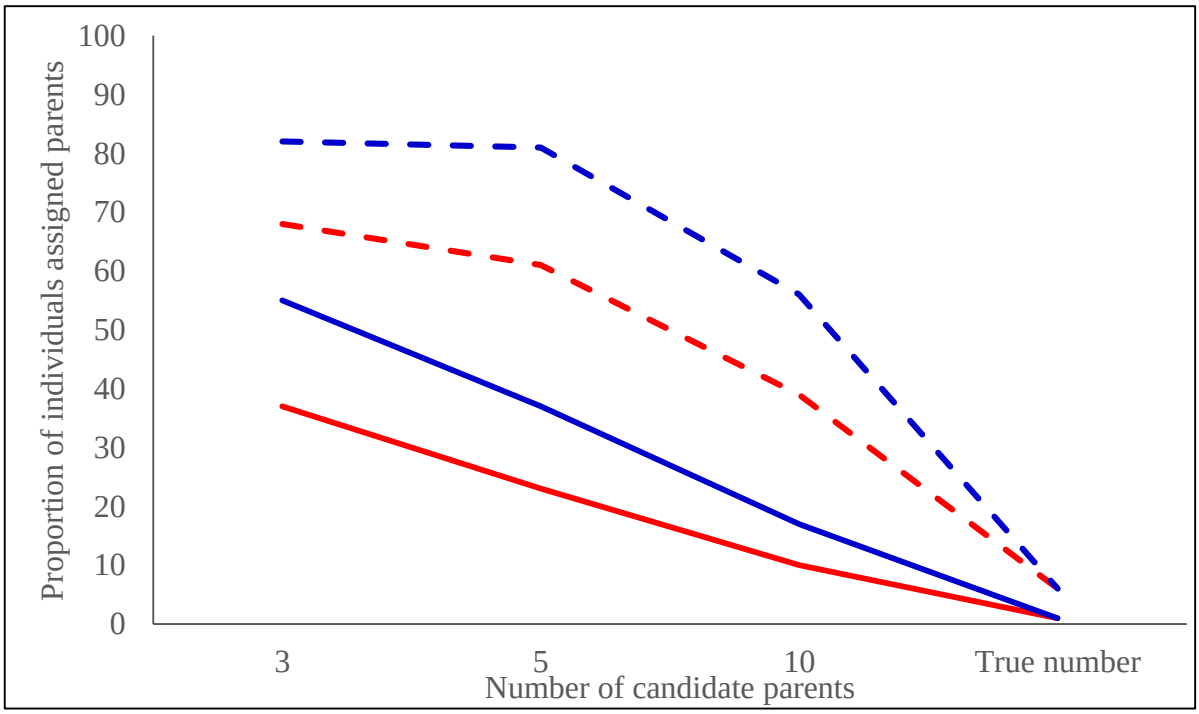

Figure 4.2 Comparison of the effect of varying numbers of candidate parents per individual on the proportion of individuals expected to have parentage assigned. Numbers of candidate parents vary from three, five, ten, or the 'true number' of the maximum of 85 candidate fathers or 61 candidate mothers included in CERVUS analysis for each individual. Red represents results of simulations of maternity analysis, while blue represent those for paternity analysis. Filled lines represent the proportion of individuals expected to have parentage assigned with 95% confidence, dashed lines represent that for 80% confidence.

Table 4.5 Results of maternity and paternity assignment when the genotype of the second parent was unknown. Maternity assignment was performed for 61 candidate mothers, with an estimated 65% of true mothers sampled. Paternity assignment was performed for 85 candidate fathers, with 83% of true fathers estimated to have been sampled.

	95% confidence		**80% confidence**	
	Critical ΔLOD	**Assignment rate (expected)**	**Critical ΔLOD**	**Assignment rate (expected)**
Maternity	4.66	2% (1%)	2.69	3% (6%)
Paternity	4.64	2% (1%)	2.35	5% (6%)

Maternity analysis in CERVUS indicated the social mother as the most likely mother when the genotype of the social father was unknown for 26 individuals, but with 95% confidence for only one individual, and 80% confidence for one additional individual. When the genotype of the social father was known, the social mother was identified as the most likely mother for 33 individuals, including five with 95% confidence, and a further four with 80% confidence. For the 21 individuals with at least a single locus mismatch, the social mother was assigned as most likely true mother when the paternal genotype was unknown for seven individuals, although the confidence of assignment was below 80% for all seven individuals. When the paternal genotype was known, three individuals with at least a single mismatch were assigned the social mother, one of which was assigned with 95% confidence. None of the individuals with mismatches to the social mother were assigned the social mother as most likely candidate mother. Across all 61 offspring, there was a mean of 8.9 ± 0.498 candidate mothers per individual with positive LOD scores.

For analysis of paternity, ten individuals were assigned the social father as most likely father when the maternal genotype was unknown, but only two of these were assigned with 80% confidence. When the genotype of the social mother was known, 22 individuals were assigned the social father as most likely genetic father. Six of these assignments were made with 95% confidence, and a further three with 80% confidence. Of the 21 individuals with at least a single mismatch to the social parents, the social father was assigned as the most likely father for one individual when the genotype of the social mother was unknown (this individual only contained allelic mismatches to the social mother), and for one individual when the genotype of the social mother was known. However, both assignments were made with less than 80% confidence. The average number of candidate fathers per individual with positive LOD scores was 14.3 ± 0.722. One putative father-offspring pair assigned with 80% confidence contained identical genotypes as listed in Table 4.3 (individual B109648, putative father B81170).

Of 38 individuals that were not assigned the social father as the most likely father when the genotype of the social mother was known, GPS data from nesting locations of the assigned father was recorded for 25 individuals. Distance of the natal site of the offspring to the nesting site of the assigned father ranged from 17 - 698 m (mean ± SE = 289.8 ± 34.23 m). Between assigned father-offspring pairs, mean relatedness was equivalent to that of a first order relationship (r_{xy} = 0.554 ± 0.031), compared to that between offspring and social fathers, r_{xy} = 0.364 ± 0.041.

4.4.5 Heterozygosity, relatedness, and extra-pair paternity

Individual heterozygosity standardised for the number of loci genotyped in parental black robins ranged from 0.508 to 1.692. Average heterozygosity of mothers of EPP progeny (Group 4 and 5 offspring; n = 9) was 1.062 ± 0.084 compared to that of mothers producing offspring with no mismatches (Group 1 offspring; n = 18) with mean heterozygosity of 0.880 ± 0.079, although this was not significantly different following Mann-Whitney U tests (two-tailed test, U = 83.5, P = 0.224). There was also no significant difference in heterozygosities between EPP offspring (Group 5, mean = 1.101 ± 0.083) and non-EPP offspring (Group 1, mean = 0.995 ± 0.049, two-tailed test, U = 212, P = 0.312).

Mean relatedness between the social father and EPP offspring (Group 4 and 5 offspring) was -0.023 ± 0.090, and was significantly lower than that of EPP offspring and putative fathers identified by parentage analysis (r_{xy} = 0.53 ± 0.043 (one-tailed test, U = 6, P = <0.001)). Between social fathers and offspring with no mismatches, r_{xy} = 0.493 ± 0.030, which was significantly greater than that of relatedness between social fathers and EPP offspring (one-tailed test, U = 24, P = <0.001).

Mean relatedness between parent-pairs was 0.018 ± 0.060. Of 35 parent-pairs, four pairs had a relatedness value equal to or greater than that of a first-order familial relationship, and a further four pairs had relatedness values greater than that of a second-order relationship. Among the nine parent-pairs with extra-pair progeny (Group 4 and 5 offspring), relatedness was not significantly different to that between pairs that produced only Group 1 offspring (Group 4 and 5 r_{xy} = 0.079 ± 0.129, Group 1 r_{xy} = 0.016 ± 0.062, two-tailed test, U = 98, P = 0.446). Relatedness between all social full-siblings (39 pairs) was r_{xy} = 0.417 ± 0.049. Mean relatedness between pairs of siblings in family groups containing only Group 1 individuals was r_{xy} = 0.521 ± 0.074, and was not significantly different from mean relatedness between pairs of EPP (Group 4 and 5) progeny and their social siblings (r_{xy} = 0.324 ± 0.072, two-tailed test, U = 56.5, P = 0.979). Of 39 sibling pairs, only twenty pairs had relatedness values equal to or greater than that of a first-order familial relationship ($r_{xy} \geq 0.5$).

4.5 Discussion

Conservative estimation of extra-pair paternity at a rate of 14.3% in the socially monogamous Chatham Island black robin is considerably higher than that measured in the New Zealand robin where an EPP rate of 1.9% was detected from microsatellite genotyping of 198 offspring and 54 breeding pairs (Taylor *et al.* 2008). The rate of EPP in the Chatham Island black robin has been assumed to be very similar to that of the New Zealand robin for analyses of inbreeding and inbreeding depression (Kennedy *et al.* 2014; Weiser *et al.* 2016). The low level of genetic diversity found in the black robin, and the identification of four pairs of individuals with identical genotypes indicates that some individuals that are the product of EPP may go undetected by chance, if the true genetic father has a number of common alleles

that are also found in the social father. This is supported by the relatively low exclusion probability of 0.763 for one parent when the other is unknown, and 0.946 when the other parent is known. In comparison, other studies of EPP in birds have consistently achieved a probability of exclusion ≥ 0.98 (e.g. Charmantier and Blondel (2003); Foerster *et al.* (2003); Li *et al.* (2009); Rosenfield *et al.* (2015); Sakaoka *et al.* (2014); (Taylor *et al.* 2008); Winternitz *et al.* (2015)). Therefore, the rate of EPP of 14.3% may be an underestimation of the true rate of EPP in the population.

The detection of individuals that were mismatched to either or both parents at two or more loci, and one individual that was mismatched only to the social mother may indicate a baseline of error within the results. Error may be due to genotyping error (which could account for approximately 38 errors across all 176 individuals genotyped), or sampling or recording error. However, due to the low level of genetic diversity and high level of inbreeding in the population, some of these errors may not result in mismatches between parents and offspring, and so may go undetected. Alternatively, individuals with mismatches to both or either parents, or to only social mother, may indicate that there is a very low frequency of intraspecific brood parasitism or quasi-parasitism occurring in the black robin population. Intraspecific brood parasitism is an alternative female reproductive strategy, whereby a female may increase her fitness by laying eggs in nests of other pairs within the same species, reducing her parental cost at the expense of the host pair (Yom-Tov 1980). Quasi-parasitism occurs when a male mates with a female outside of the social pair, and this extra-pair female lays the resulting eggs in the nest of that male and his social partner (Griffith *et al.* 2004). If IBP is occurring, some offspring in the nest would not be related to either social parents, while if quasi-parasitism is occurring, all offspring will be related to the social father, but some will not be related to the social mother. Although intraspecific brood parasitism is less common than EPP, and quasi-parasitism is very rare (Griffith *et al.* 2002; Yom-Tov 2001), the possibility of very low levels of brood parasitism in the Rangatira population cannot be rejected. Such brood parasitism may be more common at high breeding densities, as there are more nearby nests available for females to parasitise (Yom-Tov 1980).

In their comprehensive review of EPP analysis, Griffith *et al.* (2002) recommended the use of sample sizes of more than 200 offspring when examining EPP to reduce the rate of error.

However, a meta-analysis of EPP in 33 bird species found analyses used sample sizes of 13 - 202 individuals, and 5 - 21 microsatellite loci (Arct *et al.* 2015). While the current study is limited by the small population size and low breeding rate of the Chatham Island black robin, this analysis is well within the typical range in terms of sample size and number of loci, and comprised an estimated 42% of all offspring produced during the 2010 and 2011 breeding seasons (144 offspring recorded). The relatively low level of genetic diversity within the sampled population limits the power of parentage assignment, but is sufficient to identify many mismatches in genotype between social fathers and offspring.

The reason as to why black robin females engage in extra-pair copulations remains unknown, as no correlations between EPP and relatedness of breeding pairs or heterozygosity of parents or offspring have been detected. Low power of the non-parametric Mann-Whitney U test, combined with small sample sizes may have reduced the ability to detect significant differences in heterozygosities or relatedness. As such, there currently is no evidence that EPP has evolved as a strategy whereby females derive indirect benefits through avoiding inbreeding and the associated negative fitness effects, or maximising genetic diversity offspring. Further analyses of these measures across a larger number of family sets, and data from the second population on Mangere Island may assist in inferring such a pattern. Furthermore, collection of data pertaining to male body size, territory size and quality, and male and female age, may allow testing for correlations between EPP and such ecological data, potentially identifying direct benefits of EPCs to females. Comparison of rates of EPP and breeding densities between island populations may detect an effect due to differences in breeding densities (Griffith *et al.* 2002). Comparisons of fitness-related traits in EPP offspring may also detect indirect benefits of EPCs to females, whereby EPP offspring may have greater survivorship, longevity, or reproductive output than those offspring resulting from monogamy (Szulkin *et al.* 2013).

While EPP was detected, the number of assumptions surrounding assignment of paternity in CERVUS limited the confidence in results of assignment. Power to identify the genetic father of extra-pair paternity was limited due to low genetic diversity within the population, and further reduced due to uncertainty surrounding the number of candidate fathers. Nevertheless, social fathers were rejected as potential candidate fathers with positive LOD scores for all

EPP offspring. Although male dispersal between breeding seasons is uncommon, they can disperse over considerable distances (up to 690 m recorded; M. Massaro, personal communication). In addition, territory size on Rangatira Island can range from 0.85 ha to 8.36 ha (M. Massaro, personal communication). While nine of the most likely candidate fathers could be rejected as true fathers as they hatched in the same year as their putative offspring, no other candidate fathers could be rejected as true fathers, as distances between nesting site of the putative father and natal site of the offspring did not exceed 700 m for all pairs assessed. Five putative fathers were recorded to be nesting within 100 m of their putative offspring, and as such, are more likely to be the true father than other candidate males with positive LOD scores nesting at greater distances from the natal site. Typical courtship behaviours have been observed between males and females at neighbouring nest sites, including feeding of females by extra-pair males (M. Massaro, personal communication), which suggests that EPCs may be occurring between birds at neighbouring nest sites, supporting the hypothesis that geographically close males are more likely to be the genetic fathers of EPP offspring.

As EPP regularly occurs in socially monogamous species (Griffith *et al.* 2002), and rates can differ greatly between closely related species, assumptions based on EPP rates of closely related species may not account for population processes occurring in the species of interest. Such assumptions should be made with care, as they may substantially bias results of relatedness measures or effective population size estimates (Reid *et al.* 2014).

Detection of EPP in the Chatham Island black robin may have implications for future viability of the population. If the occurrence of EPP results in a bias in individuals producing offspring, such that fewer males are successfully breeding than would appear from observational data, the effective population size may be smaller than expected. The effective population size is the size of a population that would lose genetic diversity due to random drift at the same rate as the actual population (Frankham 1995; Wright 1938). If the effective population size is lower than would be predicted from social data, the population on Rangatira Island may be continuing to lose genetic diversity due to genetic drift at a higher rate than would be expected for the current population size. Loss of genetic diversity may limit the ability of the population to adapt to changing conditions (Allendorf *et al.* 2013;

Frankham 2005). Furthermore, if fewer individuals are contributing to the gene pool, the level of inbreeding in the population may be increasing, and consequently population fitness may be declining due to inbreeding depression. Reductions in fitness may result in declines in population size, further increasing the strength of genetic drift (Keller & Waller 2002). Alternatively, if females are engaging in copulations with both the social partner and additional males, the effective population size may be larger, and females may be producing more genetically diverse offspring. Therefore it is important to establish what effect the rate of extra-pair paternity has on the number of successfully breeding individuals in the population.

In addition, the identification of EPP in this population may have implications for management decisions based on social pedigrees constructed from observational data (Pemberton 2008; Reid *et al.* 2014). Following the population bottleneck, a pedigree of all breeding individuals was constructed from observational data (Massaro *et al.* 2013a). The occurrence of undetected EPP indicates that there is a level of error in this pedigree, and so management decisions and inferences based on such pedigrees should be regarded with caution (Firth *et al.* 2015; Pemberton 2008). If future conservation management is to include translocations of individuals, either between islands, or to establish a new population, the use of molecular methods to infer genetic relatedness is advisable, rather than basing decisions on observational pedigree data. Selection of individuals for the establishment of a third population based on the pedigree may inadvertently result in closely related individuals selected among founders, and so would reduce the success of such establishment in two ways. Firstly, such closely related individuals would be more likely to carry similar genotypes, thereby reducing overall genetic diversity in the new population below the maximum possible, and secondly, the rate of inbreeding in the new population may increase, resulting in significant reductions in population fitness due to inbreeding depression (Keller & Waller 2002). Therefore, using molecular methods to infer relatedness between individuals is strongly recommended as the basis for selection of individuals for such future translocations.

The detection of a moderate rate of extra-pair paternity in this socially monogamous species where no evidence of EPCs has been recorded despite the collection of observational data

over more than thirty years is a reminder that species should not be assumed to be monogamous without closer investigation. Even closely related species may display dramatically different breeding systems, which may arise due to their different demographic histories or ecologies. The detection of previously unrecognised EPP, and a potential low frequency of intraspecific brood parasitism in the Chatham Island black robin requires the use of molecular techniques to estimate relatedness in this species. Such methods are recommended over the use of the observed pedigree to provide greater accuracy of analysis and to improve success of conservation management of this endangered species.

Chapter Five

General Discussion

Species confined to small islands are expected to display lower genetic diversity and have higher rates of inbreeding compared to similar species on the mainland (Frankham 1997, 1998). Both low genetic diversity and high rates of inbreeding can make populations more vulnerable to extinction. Low genetic diversity may limit the ability of such populations to adapt to altered conditions (Allendorf *et al.* 2013), while high rates of inbreeding leads to inbreeding depression that may reduce population fitness (Keller & Waller 2002). A history of small population size and intense inbreeding has resulted in low minisatellite diversity (Ardern & Lambert 1997) and varying effects of inbreeding depression (Kennedy *et al.* 2014; Weiser *et al.* 2016) in the Chatham Island black robin *Petroica traversi*, once considered the world's most endangered bird (Butler & Merton 1992).

5.1 Findings from this study

5.1.1 Primer development

To investigate population genetic structure and diversity within and between the two island populations of black robins, I designed microsatellite primers specifically for the black robin using Illumina sequencing (Chapter Two). Previously, only three microsatellite loci were found to be polymorphic in the black robin, and these loci were insufficiently informative to assess genetic diversity and population structure (Cubrinovska *et al.* 2016). Following Illumina sequencing, 40 primer pairs were selected for testing, from which 11 loci amplified consistently and were polymorphic. In addition, testing of primer pairs that amplified in the black robin revealed 17 loci were polymorphic in the Chatham Island tomtit, the sister-species of the black robin. Direct comparison of genetic diversity between these two species was possible as eight loci were polymorphic in both species.

5.1.2 Genetic diversity of the Chatham Island black robin

Using the 11 loci polymorphic in the black robin, the three loci previously found to be polymorphic (Cubrinovska *et al.* 2016), and an additional locus isolated from the South Island robin (Townsend *et al.* 2012), I was able to answer several questions relating to the population genetics of the black robin. Firstly I investigated how genetically diverse each black robin population is (Chapter Three). Genotyping of 145 black robins from the two island populations found low levels of allelic diversity (2.67 alleles per locus) and expected heterozygosity (0.408) in the black robin. This level was similar to other New Zealand bird species, and threatened passerine species worldwide. Furthermore, the smaller black robin population on Mangere had lower genetic diversity than that on Rangatira. This indicates that loss of diversity due to genetic drift may be continuing within this small population. While the tomtit populations were found to be more genetically diverse than the black robin populations, the smaller Mangere population also exhibited relatively low diversity, and while the tomtit is ranked as a species of Least Concern by the IUCN (BirdLife International 2016a), genetic diversity of the tomtit was similar to that of other threatened passerines. This should generate concern, as low genetic diversity may increase the extinction vulnerability of the tomtit.

5.1.3 Genetic differentiation between island populations

The second key question that I addressed in my thesis was whether the Mangere and Rangatira populations of robins differ genetically (Chapter Three). Analysis showed that the Mangere and Rangatira black robin populations are substantially differentiated from one another, and that there is no evidence of current gene flow through dispersal between these island populations. While historic natural dispersal cannot be completely ruled out, it is more likely that alleles occurring at low frequency in the Rangatira population (but which are common in the Mangere population) are remnants from founding individuals that were introduced from Mangere to Rangatira in the 1980s. The two black robin populations have only been isolated for 26 years, but genetic drift appears to have had strong, independent effects on these small populations. Similarly, the Rangatira and Mangere tomtit populations were found to be differentiated from one another, to a similar degree as the black robin populations. While it may be possible for tomtit individuals to disperse between the islands, using the third population on Pitt Island as a stepping-stone, no evidence of current gene flow was found between Mangere and Rangatira tomtit populations. The level of differentiation

between island populations combined with the lack of evidence of gene flow via dispersal suggests that the tomtit populations may have been isolated from one another for as long as the robin populations have been isolated.

5.1.4 Population substructure on Rangatira Island

The presence of distinct forest habitats (Woolshed Bush and Top Bush) on Rangatira Island in combination with differences in breeding densities has led some researchers to suggest that there may be population substructuring within the black robin population on Rangatira Island (Kennedy 2009). My genotyping results, in combination with data on sampling locations of individuals allowed me to test whether there is any substructure within the Rangatira black robin population (Chapter Three). I found no evidence of a substructure within the Rangatira population, and therefore confirm that it is unnecessary to treat the two bush areas as separate populations in any future analyses.

5.1.5 Breeding system of the black robin

The microsatellite loci used displayed a sufficient level of variation to confirm whether the black robin was both socially and genetically monogamous. Very low levels of extra-pair paternity (EPP) have been found in a close congener, the New Zealand robin *Petroica australis* (Taylor *et al.* 2008), and no evidence of EPP has ever been recorded over thirty years of data collection in the Chatham Island black robin (Butler & Merton 1992; Kennedy 2009). However, EPP can remain undetected in observational studies, and is a very common strategy among birds (Griffith *et al.* 2002). I found that although genetic monogamy is the norm in the black robin, EPP occurred at a rate of 14% in the families assessed, with EPP offspring comprising 8.3% of all offspring (Chapter Four). In addition, the results of this study could not reject the possibility of a very low level of intraspecific brood parasitism or quasi-parasitism. The detection of EPP and brood parasitism casts doubt on the known pedigree based on observational data, as clearly some individuals are not offspring of the social father, or in some cases, may not be the progeny of either social parent.

5.2 Recommendations for management

5.2.1 Translocation: Assisted gene flow

Based on the results of this study, I strongly recommend that translocations of black robin individuals between the existing island populations be carried out, as a method of genetic restoration (Weeks *et al.* 2011). Reciprocal translocations simulate gene flow between the isolated island populations, bolstering the level of genetic diversity within each population. The very small population on Mangere may be continuing to lose genetic diversity due to genetic drift, and the introduction of individuals from Rangatira Island, containing alleles rare to the Mangere population, may minimise future loss of diversity. Furthermore, as the population on Mangere has remained very small, the rate of inbreeding may be increasing in this population. Translocations of birds between islands may aid in reducing the rate of inbreeding, as highly related birds within the Mangere population can be translocated to Rangatira Island, and be replaced by relatively unrelated individuals from Rangatira Island. Modelling to determine sufficient numbers of individuals and the frequency of such translocations will be required to determine levels that will minimise risks to the existing populations. The accuracy of such modelling will be greatly improved by incorporating the results of the assessment of allelic diversity and heterozygosity from this study.

5.2.2 Translocation: Establishment of a third population

In addition to reciprocal translocations between black robin populations, I further recommend that the establishment of a third population be urgently reconsidered. Although reciprocal translocations may be sufficient to limit future loss of diversity from these populations, we should not rely on conditions remaining the same. Such small populations are highly vulnerable to altered conditions, such as the introduction of novel disease (Blackburn *et al.* 2004; McCallum 2008) that may destabilise the population. Such impacts may rapidly reduce population size and increase loss of diversity and inbreeding once more. As insurance alone, a third population is vital. Furthermore, establishing a third population will allow population expansion beyond the maximum currently possible, and over time will provide a third population to engage in translocations to improve overall diversity and minimise inbreeding. Failure to successfully establish a population on Pitt Island from 2002 - 2004 appears to have detracted from further attempts, and unfortunately the cause of this failure was not clearly identified (Kennedy 2009). Careful evaluation of the number of individuals initially required,

with selection based on relatedness of individuals within their current population will be necessary to minimise the founder effect (Jamieson 2011; Miller *et al.* 2009; Tracy *et al.* 2011) and maximise the success of such establishment. To capture at least 95% of the genetic diversity of the current population, between twenty and fifty individuals are typically required (Weeks *et al.* 2011). Individuals from both the differentiated Mangere and Rangatira populations should be included to capture the maximum diversity of the species. The finding of no substructure within the Rangatira population means that sourcing individuals from both bush areas is not required. Close monitoring during the initial release, perhaps with supplementary feeding and provision of nest boxes will be required, and careful recording of the fate of each translocated individual is necessary to further maximise success. While the costs of such a project may appear high, the benefit of establishing a third population, and thereby ensuring the prolonged survival of this iconic endangered species, will vastly outweigh these costs. Pitt Island remains a location with great potential for translocation, particularly if predator removal is undertaken.

An alternative site is Little Mangere Island, from which the seven remaining individuals were removed and established on Mangere Island in the 1970s (Butler & Merton 1992). As the last remaining wild refuge of the black robin, the return of the black robin would have historic and cultural significance. Although the black robin is averse to flying across open areas, it is hypothesised that the Mangere and Little Mangere populations may once have acted as a single populations, with 260 m between the closest forest edges potentially being within the range for dispersal (Kennedy 2009). If this was the case, and a population was to be re-established on Little Mangere Island, the two islands combined could support approximately eighty birds, and dispersal between these islands may reduce the rates of inbreeding and effects of genetic drift on such isolated small populations. While establishment on Little Mangere Island would have some benefits to the species, establishing a population on Pitt Island that could grow substantially beyond that of a population on Little Mangere is preferable, and would maximise these benefits.

5.3 Future directions

5.3.1 Use of the developed primers

The primers developed in this study can be used for further analyses of the tomtit populations, and are likely to prove useful in other species within the Petroicidae family. In addition, many more sequences were obtained from Illumina sequencing than were assessed for amplification and polymorphism in Chapter Two, and so more polymorphic loci are likely to be available for analyses of the tomtit, or other members of the Petroicidae family. The use of Illumina sequencing allows preferential selection of microsatellite loci that are more likely to be polymorphic (due to the large number of perfect repeats), both in the target species, and in non-target species.

5.3.2 Chatham Island tomtit

Further research into genetic diversity and structure of the Chatham Island tomtit populations is recommended, and in particular, inclusion of sampling of individuals on Pitt Island. This will allow greater accuracy by measuring the level of diversity across the entirety of the species range. This will allow more in-depth investigation of dispersal between islands, to determine whether the Pitt Island population may act as a stepping-stone, or whether there may be any source-sink dynamics between the three populations. If gene flow is occurring between these populations, the tomtit may be less vulnerable than it appears from this preliminary study. The tomtit is regarded as a species of Least Concern (BirdLife International 2016a), but the apparent isolation of at least two of its three populations may have resulted in strong genetic drift acting on the relatively small populations, and consequently these smaller populations may be continuing to lose genetic diversity. The results of the current study place the tomtit among both threatened New Zealand birds and threatened passerines worldwide in terms of microsatellite diversity. Conservation management should not merely rescue species from extinction, but should aim to prevent species from becoming vulnerable in the first place.

5.3.3 Breeding systems

Further investigation into EPP and potential intraspecific brood parasitism in the Chatham Island black robin are also recommended. Analysis including a larger number of family groups may allow further clarification of how frequently intraspecific brood parasitism is in this species. With a greater number of family groups included, more robust comparisons of

heterozygosity and relatedness can be performed with larger sample sizes, which may help to determine the reasons for the evolution of this strategy in black robins. The collection of ecological data pertaining to male size and territory quality may reveal an ecological explanation for EPP (Griffith *et al.* 2002; Westneat *et al.* 1990; Westneat & Stewart 2003). Recording of the social pedigree from Mangere Island would allow a similar analysis to be conducted for that population, and may reveal differences due to population size, breeding density, or rates of inbreeding. Similar analyses could be carried out for the tomtit populations if social pedigree was recorded, and would allow comparison of the rate of EPP between species within the Petroicidae family, which may reveal differences due to population size, historic demography, or level of endangerment.

5.4 Conclusions

The results of this study show that the Chatham Island black robin populations have lower diversity than those of the closely related tomtit. Levels of genetic diversity are similarly low in other threatened species worldwide, suggesting that vulnerability to extinction is high. The two populations of robins are genetically differentiated, despite a short period of isolation. As strong genetic drift is likely to be continuing, particularly in the very small population on Mangere Island, I recommend the occasional translocations of individuals between island populations to mitigate this loss of diversity. I further recommend that translocations of individuals from both existing populations be used to establish a third population, to allow the species to increase in size beyond what is currently possible. These translocations will improve evolutionary potential and decrease inbreeding depression. As the rate of EPP is higher than expected, this has implications for the accuracy of the observed pedigree, and so genetic measures of relatedness should be used to assess individuals to be selected for these translocations.

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Appendices

Appendix 1 Manuscript accepted for publication in Conservation Genetics Resources, March 2016, based on material presented in Chapter Two.

Forsdick, N.[1], Lindgreen, S.[1,2], Massaro, M.[1,3], Hale, M.L.[1] Isolation and characterisation of microsatellite loci for the Chatham Island black robin *Petroica traversi.* Microsatellite Record in *Conservation Genetics Resources* (in press).

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Methods Text

DNA was extracted from blood spots using a PureLink™ Genomic DNA Mini Kit (Invitrogen). DNA quantity was low, so extracted DNA from three individuals was pooled to ensure sufficient quantity (> 2.5 µg) for next generation (Illumina) sequencing, conducted by New Zealand Genomics Ltd. A MiSeq 250 bp paired-end run, with 550bp insert size, produced 9,596,842 raw read pairs. PAL_finder (v. 0.02.04) (Castoe et al. 2012) identified 50,795 reads as containing potential microsatellites. PAL_finder was run with default settings except for the primer minimum temperature (PRIMER_MIN_TM=55) and the minimum number of n-mer repeats detected (2mer - minimum 10 repeats; 3mer - minimum 8 repeats; 4mer - minimum 7 repeats). 10,126 reads containing microsatellites with sufficient overlap (at least 11 nucleotides), were then collapsed using AdapterRemoval 2.0 (Lindgreen 2012) and primers designed using Primer3 (Koressaar & Remm 2007; Untergasser *et al.* 2012). Primers for forty microsatellite regions were selected for testing of amplification and polymorphism (GenBank accession numbers KU194428-KU194467). M13 tags (5'-TGTAAAACGACGGCCAGT) were added to the forward primers for universal fluorescent dye labelling (Schuelke 2000). PIGtails (GTTTCTT) were attached to the reverse primers to reduce adenylation (Brownstein *et al.* 1996).

Polymorphic loci were amplified in 15 µl reactions containing 0.5 µl of genomic DNA, 1 x NH_4 reaction buffer (Bioline), 2 mM $MgCl_2$, 0.08 mM dNTPs, 0.083 µM of forward primer, 0.33 µM reverse primer and 0.33 µM fluorescently labelled M13 primer (5'-TGTAAAACGACGGCCAGT labelled with 6-FAM, NED, VIC, or PET, Applied Biosystems), and 0.6 U BIOTAQ DNA polymerase (Bioline). One of two thermocycle protocols was used: either a standard three-step protocol, or a touchdown (TD) protocol (See Table 1). The standard three-step protocol consisted of: 95°C for 12 min, 10 cycles of 94°C for 15 s, annealing temperature (T_A)°C (Table 1) for 30 s, 72°C for 30 s, followed by 30 cycles of 89°C for 15 s, T_A°C for 30 s, 72°C for 30 s, then a final extension of 72°C for 10 min. If initial $T_A \geq 54$°C, the protocol was altered to 95°C for 12 min, 10 cycles of 94°C for 15 s, T_A° for 30 s, 72°C for 30 s, followed by 30 cycles of 89°C for 15 s, 53°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 10 min. This improved annealing of the M13 primer (Schuelke 2000), except when using PT2, PT24, and PT27, where the original protocol was used. The touchdown protocol was used for loci that amplified inconsistently at a single temperature, and consisted of: 95°C for 15 min, 10 cycles of 94°C for 15 s, T_A° C (from 62°C to 53°C, decreasing 1°C per cycle) for 30 s, 72°C for 1 min, then 25 cycles of 94°C for 15 s, 52°C for 30 s, 72°C for 1 min, followed by a final extension step at 72°C for 15 min. Genotyping was performed on an ABI Prism® 3130xl Genetic Analyser (Applied Biosystems). Allele sizes were scored visually using GeneMarker (v.2.20; SoftGenetics).

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Table 1 Characterisation of 20 microsatellite loci developed for *P. traversi* (black robin) and cross-amplification in *P. macrocephala chathamensis* (Chatham Island tomtit). T_A = Optimum annealing temperature, n = number of individuals genotyped, N_A = number of alleles, H_O = observed heterozygosity, H_E = heterozygosity expected under Hardy-Weinberg Equilibrium (HWE). TD = touchdown, M = locus monomorphic, D = locus did not amplify well, * = Locus significantly out of HWE.

Locus	**Primer sequence**	**Repeat motif**	**T_A (°C)**	**Allele size range (bp)**	**Black Robins**				**Tomtits**			
					n	**N_A**	**H_O**	**H_E**	**n**	**N_A**	**H_O**	**H_E**
PT1	F: GGCCCATCTTTGAAGGTTCT	AAAG(23)	TD	322-360	30	3	0.567	0.660		D		
	R: TCCAAAGTATCCCACCAGCA											
PT10	F: CCTGCTCAAACTGAAGTCCG	TTC(20)_TCC(11)	54	325-338	30	2	0.300	0.381	30	2	0.400	0.325
	R: TGACATCCCCTTTGTGATGC											
PT12	F: CACAGGGGAGGACTTGAGAA	ATAC(11)_AT(15)	TD	192-198		M			30	4	0.567	0.610
	R: AGTTCAGGTCTGCTTTGTACA											
PT18	F: TCCCACCCTGTTCTCCATTT	AGT(21)	56	220-229	30	2	0.467	0.364	30	3	0.600	0.677
	R: TTAGGTGCCGTGACTTCTCA											
PT19	F: AGCAGGCATTTTCAGCACTC	AGTG(17)	56	173-201		M			30	7	0.833	0.812
	R: GCAACCCCACAAAACCTGAT											
PT2	F: GCTATTTGTGCAGGGAAGTG	AAAG(23)	56	202-226	30	2	0.367	0.305	30	6	0.700	0.754
	R: TGTGCCAGATTTTCCACAGC											
PT24	F: GCCAGCAGGTAAGTTGTGCT	ATC(24)	60	119-153		M			29	3	0.200	*0.640
	R: CCTCCCTTCTCCCTGTCTCT											
PT25	F: TAAAGGGAGCAAAGGAGGCA	ATGG(17)	58	150-208		M			29	10	0.586	0.727
	R: TGTCCAAAGTCCCTCTCCAG											

Table 1 continued.

Locus	Primer sequence	Repeat motif	T_A (°C)	Allele size range (bp)	Black Robins				Tomtits			
					n	N_A	H_O	H_E	n	N_A	H_O	H_E
PT26	F: TGCCATGCTTATTCTGGGGA R: CTATGGGAGTGTGCCTGTAG	ATGG(17)	TD	196-238	30	2	0.033	0.033	30	4	0.467	0.681
PT27	F: ACATACCACCTGCCACTTCA R: TGTCTGGGCTTTAATGTCTCAC	ATT(28)	60	214-240	30	3	0.300	0.362	28	7	0.714	0.690
PT35	F: CTGAAAGAGGGCACAGCTTC R: GAGATGCACTTCTTTGGCGA	AC(20)	56	83-88		M			30	3	0.467	0.453
PT37	F: TCTTGGTGGGGATCTACACAC R: ACTTCCCATGGCAGAACAGT	AC(21)	58	121-141	30	2	0.300	0.305	30	2	0.400	0.506
PT38	F: CCTGCCCAGACCAACTCT R: AAATGAATCCTCGCTGTCCA	AC(21)	TD	101-105	30	2	0.233	0.259	8	D		
PT39	F: CCATGCAACTACGGGTGTTT R: TGTCTGAGAACCCCAGAAGG	AC(21)	48	99-109	30	2	0.367	0.463	30	2	0.367	0.345
PT4	F: TATCTCCACATGGTGCAGGC R: TGCTATGGTTTTATGCCCTGG	AATG(14)	54	281-300		M			29	6	0.897	0.753
PT40	F: ACTTTGAAATACTCTCGAGGGC R: TGGAATCCATTTTGTGCAAT	AC(25)	48	132-143	30	2	0.557	0.508		M		
PT5	F: GTCTCTGGTGAGTCCTGGGG R: GGGGTTTGGAACAATCATCC	AGGG(10)_ATGG(10)	54	246-254		M			30	3	0.433	0.515

Table 1 continued.

Locus	Primer sequence	Repeat motif	T_A (°C)	Allele size range (bp)	Black Robins n	Black Robins N_A	Black Robins H_O	Black Robins H_E	Tomtits n	Tomtits N_A	Tomtits H_O	Tomtits H_E
PT6	F: CAACCATGTGAACGGTCTGC R: AAAGGAGTGGGATTTGGAAGC	AGT(21)	54	268-300		M			30	5	0.467	0.563
PT7	F: GCTCACCTTTTACAATCCTCTGC R: CCTGCTGCTGTTTAGAAGCC	ATCT(15)	54	265-288	30	3	0.600	0.594	30	5	0.800	0.755
PT9	F: CCTCTTGGAGAGGTTCTGCG R: GTGATGAGTCAGCTCCAGCG	TGC(23)	TD	285-304		M			29	4	0.300	*0.488

Appendix 2 Table of allele frequencies for 15 polymorphic microsatellite loci in the Chatham Island black robin. Results are based on genotyping of thirty individuals were genotyped from Mangere Island, and 115 individuals from Rangatira Island.

Locus	Allele	Mangere	Rangatira
PT1	319	0.000	0.014
	322	0.133	0.359
	338	0.000	0.005
	343	0.467	0.209
	347	0.033	0.000
	360	0.367	0.414
PT2	202	0.714	0.793
	220	0.286	0.198
	229	0.000	0.009
PT7	265	0.133	0.409
	272	0.333	0.123
	275	0.533	0.468
PT10	326	0.429	0.613
	338	0.571	0.387
PT18	220	0.362	0.305
	229	0.638	0.695
PT26	208	0.367	0.986
	212	0.000	0.005
	238	0.633	0.009
PT27	224	0.130	0.225
	233	0.870	0.757
	236	0.000	0.018
PT37	137	1.000	0.851
	141	0.000	0.149
PT38	101	0.467	0.162
	105	0.533	0.838
PT39	107	0.967	0.680
	109	0.033	0.320
PT40	132	0.667	0.554
	143	0.333	0.446
PAU26	193	0.067	0.234
	195	0.933	0.766
PCA12	100	0.367	0.426
	108	0.633	0.574
PGM1	287	0.000	0.005
	292	0.000	0.005
	294	0.900	0.750
	302	0.100	0.240
TG-02	261	0.150	0.291
	262	0.850	0.709

Appendix 3 Table of allele frequencies for 17 polymorphic microsatellite loci in the Chatham Island tomtit. Results are based on genotyping of 22 individuals from Mangere Island and thirty from Rangatira Island.

Locus	Allele	Mangere	Rangatira
PT2	209	0.295	0.267
	213	0.091	0.117
	217	0.000	0.017
	220	0.000	0.017
	222	0.273	0.333
	226	0.341	0.250
PT4	281	0.500	0.276
	285	0.024	0.017
	289	0.119	0.345
	292	0.071	0.224
	296	0.048	0.121
	300	0.238	0.017
PT5	246	0.977	0.617
	250	0.023	0.333
	254	0.000	0.050
PT6	268	0.318	0.283
	285	0.000	0.017
	288	0.659	0.600
	291	0.023	0.033
	300	0.000	0.067
PT7	265	0.045	0.217
	275	0.477	0.167
	278	0.182	0.333
	281	0.295	0.267
	288	0.000	0.017
PT9	285	0.000	0.083
	291	0.295	0.700
	300	0.000	0.100
	304	0.705	0.117
PT10	325	0.614	0.800
	328	0.386	0.200
PT12	192	0.659	0.567
	194	0.091	0.233
	196	0.250	0.150
	198	0.000	0.050
PT18	220	0.500	0.350
	223	0.386	0.333
	226	0.114	0.317

Table of allele frequencies continued.

Locus	Allele	Mangere	Rangatira
PT19	173	0.136	0.217
	177	0.159	0.017
	181	0.091	0.250
	185	0.432	0.033
	193	0.000	0.100
	197	0.182	0.250
	201	0.000	0.133
PT24	119	0.636	0.483
	147	0.023	0.300
	153	0.341	0.217
PT25	150	0.432	0.172
	167	0.023	0.034
	175	0.000	0.121
	179	0.273	0.483
	183	0.023	0.017
	191	0.000	0.052
	195	0.250	0.052
	199	0.000	0.017
	203	0.000	0.034
	208	0.000	0.017
PT26	196	0.432	0.100
	200	0.500	0.450
	208	0.023	0.317
	219	0.045	0.133
PT27	214	0.000	0.089
	217	0.000	0.071
	220	0.295	0.089
	224	0.000	0.018
	227	0.705	0.518
	236	0.000	0.179
	240	0.000	0.036
PT35	83	0.091	0.167
	86	0.909	0.717
	88	0.000	0.117
PT37	121	0.045	0.467
	123	0.955	0.533
PT39	99	0.727	0.783
	106	0.273	0.217