

Historic and contemporary levels of genetic variation in two New Zealand passerines with different histories of decline

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Abstract

We compared historic and contemporary genetic variation in two threatened New Zealand birds (saddlebacks and robins) with disparate bottleneck histories. Saddlebacks showed massive loss of genetic variation when extirpated from the mainland, but no significant loss of variation following a severe bottleneck in the 1960s when the last population was reduced from ~1000 to 36 birds. Low genetic variation was probably characteristic of this isolated island population: considerably more genetic variation would exist in saddlebacks today if a mainland population had survived. In contrast to saddlebacks, contemporary robin populations showed only a small decrease in genetic variation compared with historical populations. Genetic variation in robins was probably maintained because of their superior ability to disperse and coexist with introduced predators. These results demonstrate that contemporary genetic variation may depend more greatly on the nature of the source population and its genetic past than it does on recent bottlenecks.

Introduction

Threatened species may experience losses in genetic variation through immediate loss of alleles when population size rapidly declines and through subsequent genetic drift. Reduction in genetic variation can decrease fitness in individuals and populations (Saccheri *et al.*, 1998; Slate *et al.*, 2000; Hansson *et al.*, 2001) and reduce evolutionary potential of species, preventing them from adapting to the dynamic nature of their environment and reducing long-term survival (Burger & Lynch, 1995; Lacy, 1997). Consequently, evaluating and maintaining genetic variation in threatened species is an important goal of conservation biology.

Many studies use contemporary levels of genetic variation to evaluate the effects of recent bottlenecks, population fragmentation or drift, and then assess extinction risk and management options for threatened species. However, contemporary patterns of genetic variation may not be recent in origin and therefore may not provide an appropriate basis for these conclu-

sions. Some recent studies have included historical DNA of species in their analyses to better understand loss of genetic variation over time and clarify relationships among fragmented populations (Roy *et al.*, 1994; Nielsen *et al.*, 1999; Leonard *et al.*, 2000; Hofkin *et al.*, 2003; Miller & Waits, 2003).

Many of the studies that use historical (ancient) DNA to examine loss of genetic variation find that heterozygosity, haplotype variation and allelic diversity have decreased following population bottlenecks (Bouzat *et al.*, 1998; Glenn *et al.*, 1999; Groombridge *et al.*, 2000; Whitehouse & Harley, 2001; Larson *et al.*, 2002; Wisely *et al.*, 2002). Although such reductions in genetic variation following bottlenecks are consistent with theoretical expectations, low genetic variation in contemporary populations can also result from ancient events such as post-glacial re-colonization or bottlenecks from pre-historic human activity (Culver *et al.*, 2000; Matocq & Villablanca, 2001; Pertoldi *et al.*, 2001; Paxinos *et al.*, 2002). Similarly, long-term drift and ancient founder events may cause genetic invariance in small, isolated populations.

Small, historically isolated populations may also be more likely to survive current species declines because their isolation protects them from threats such as loss of habitat through agriculture and urbanization, exotic

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predators and infection from pathogens. Threatened species may often persist in isolation and possess low contemporary genetic variation for historical reasons, a connection that is largely overlooked. We examined the link between past and present genetic variation in two New Zealand bird species with different dispersal capabilities and histories of decline using museum DNA.

Saddlebacks (*Philesturnus carunculatus*) are members of the ancient endemic family of New Zealand wattlebirds (Callaeidae), have poor flight capability and are highly susceptible to predation by introduced mammals including rats (*Rattus norvegicus*, *R. rattus*, *R. exulans*) and stoats (*Mustela erminea*) (Higgins *et al.*, 2006). The South Island subspecies (*Philesturnus c. carunculatus*) was extirpated from the mainland by 1905, surviving only on the rat-free island of Big South Cape (939 ha) and two nearby islets of Pukeweka (2 ha) and Solomon (25 ha), all off the SW coast of Stewart Island (Hooson & Jamieson, 2003; Fig. 1). The Big South Cape group of islands had a thriving saddleback population with enough habitat to support at least 1000 birds (Hooson & Jamieson, 2004) until 1962 when rats arrived and rapidly attained plague proportions, causing a severe decline in saddlebacks. In 1964, 36 saddlebacks were caught on the Big South Cape group and became part of the first threatened species translocation carried out by the New Zealand Wildlife Service (subsequently the Department of Conservation;

Merton, 1975). The 36 birds were split between two nearby rat-free islands: Big (21 birds released) and Kaimohu (15 released). Both translocated populations established and grew, ultimately founding all living saddlebacks, now numbering approximately 1200 individuals on 15 translocated island populations located off mainland South Island (Hooson & Jamieson, 2003). No saddlebacks survived on Big South Cape, Pukeweka or Solomon Islands past the early 1970s (Merton, 1975).

There is no natural movement among the 15 present-day populations because saddlebacks are weak fliers and are not known to have self-colonized islands more than 250 m apart (Newman, 1980). However, we consider the historic populations on Big South Cape, Pukeweka and Solomon Islands to be one population because all are within saddleback flying range, theoretically enabling gene flow among the islands.

New Zealand robins (*Petroica australis*) are endemic to New Zealand but belong to the widespread Australasian robin family Petroicidae (Higgins & Peter, 2002). The South Island subspecies (*Petroica a. australis*) was formerly abundant and widespread throughout South Island and adjacent offshore islands, but the mainland population has become increasingly fragmented as a result of habitat loss and predation (Heather & Robertson, 1996; Duncan *et al.*, 1999). At present, robins are common on several South Island offshore islands and occur at lower densities

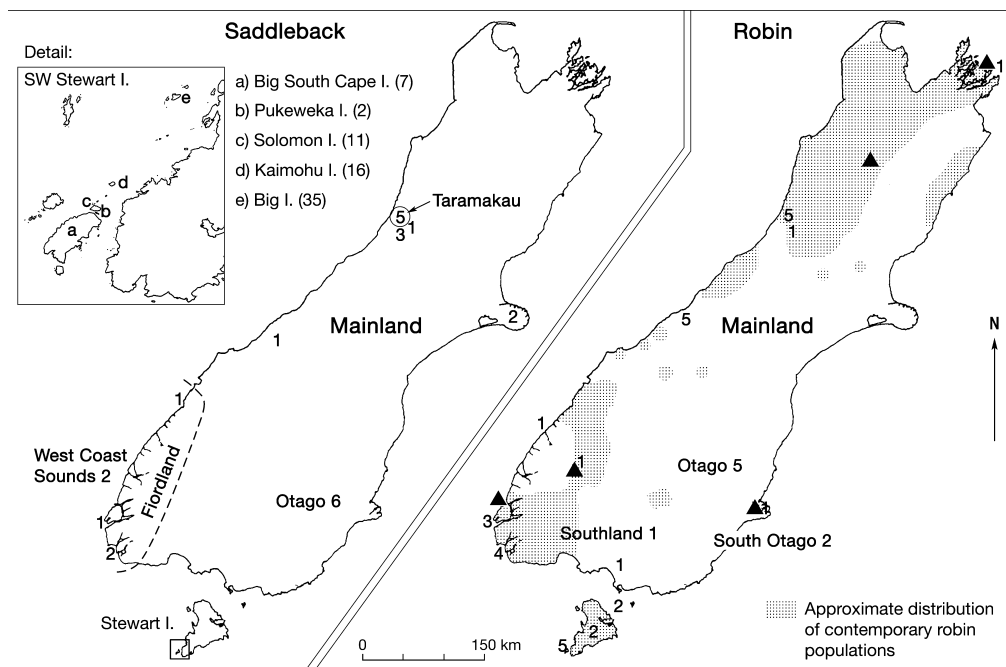


Fig. 1 New Zealand South Island sampling locations for saddlebacks (A) and robins (B). Where precise locations are unknown, the area name is given followed by sample size. Sampled contemporary saddleback populations are located on Kaimohu and Big Islands. All other saddleback samples are historic. Sampled contemporary robin populations are labelled with solid triangles and the overall present distribution is shown by stippling (based on Bull *et al.*, 1985). All other robin samples are historic. Numbers indicate sample sizes.

in disjunct mainland populations (Fig. 1; Bull *et al.*, 1985; Heather & Robertson, 1996). Robins are more capable fliers than saddlebacks and are known to have naturally colonized some islands, but do not commonly fly across large bodies of water or unforested habitat.

In comparing historic (museum) and contemporary genetic variation in these two species, we make two predictions. First, we expect to find substantially higher levels of genetic variation for historic vs. contemporary samples in both species. Second, we expect the level of genetic variation in contemporary populations of saddlebacks to be more affected than that of robins as a result of differences in dispersal capability and historical patterns of isolation.

Materials and methods

Sampling

Contemporary birds

Saddlebacks were sampled in 2005 from Big and Kaimohu Islands (Table 1; Fig. 1), the source populations of all extant saddlebacks, which presumably possess the greatest genetic variation of the 15 translocated island populations. Robin samples were collected between 2000 and 2005 as part of a larger study, which included two naturally colonized island and three mainland populations from a variety of locations (Table 1; Fig. 1).

Birds were captured in mistnets or handnets, banded with unique metal bands and sampled for a small amount of blood (100 μ L). Blood samples were obtained by

venipuncture of the brachial vein and stored at 4 °C in 1.5-mL tubes containing lysis buffer (Seutin *et al.*, 1991).

Museum birds

Toepad samples (Mundy *et al.*, 1997) were obtained from 44 saddleback and 43 robin museum specimens in nine collections worldwide (Appendix 1). Saddleback museum specimens came from several locations on mainland South Island (1877–1898, $n = 24$) and from the remnant population on Big South Cape, Solomon and Pukeweka Islands (1931–1965, $n = 20$), including five birds that died during an unsuccessful translocation to Inner Chetwode Island in 1965. We consider these five birds to form part of the prebottleneck sample because they were almost certainly conceived prior to the arrival of rats. Adult saddlebacks have an average longevity of 8 years (maximum recorded 17 years; Nilsson, 1978; Heather & Robertson, 1996; I. Jamieson, unpublished data) and all five birds were at least 2-year-old adults in 1965 (based on plumage characteristics; Higgins *et al.*, 2006). Finally, rats typically depredate nest contents and incubating females first (Lovegrove, 1996) making it very unlikely that any of the five birds hatched post-1962 when rats were present. Robin specimens from 1873 to 1955 were from several sites on mainland South Island (including Stewart Island; $n = 34$) and islets off Stewart and Queen Charlotte Islands ($n = 8$; Table 1). Curatorial staff at overseas museums were asked to follow our sampling protocol, which involved using sterile tubes, fresh scalpel blades and gloves for each specimen, and sterilizing (with bleach) scalpel handle, forceps and laboratory bench before handling each specimen.

Table 1 Sampling locations for South Island saddlebacks and robins.

Saddlebacks	<i>n</i>	Robins	<i>n</i>
Museum – Offshore Islands (1931–1965)		Museum (1873–1955)	
Big South Cape Island	7	Stewart Island	2
Pukaweka Island	2	Islets nearby Stewart Island	7
Solomon Island	11	Fiordland	10
Total	20	Otago	9
		Southland	2
Museum – Mainland (1877–1898)		Eglington Valley	1
Fiordland	6	Paringa	5
Otago	6	Taramakau	5
Banks Peninsula	2	Taiipo River	1
Taramakau	5	Pickersgill Island	1
South Taiipo River	3	Total	43
Bealey River	1		
Blue River	1		
Total	24		
Contemporary – Offshore Islands (2005)		Contemporary (2000–2005)	
Big Island	35	Naturally colonized islands	80
Kaimohu Island	16	Mainland populations	203
Total	51	Total	28

DNA extraction

Contemporary samples

DNA was extracted from blood using a Chelex resin protocol (Walsh *et al.*, 1991). Blood/lysis buffer mixture (10–100 μ L) was added to 800 μ L of Milli-Q filtered water, 8–15 g of Chelex 100 resin and 40 μ g of proteinase K. Samples were lysed overnight at 65 °C, heated to 100 °C for 10 min and then centrifuged at 20 125 g for 10 min to pellet Chelex and bound impurities. The supernatant was transferred to new tubes with a working stock kept at 4 °C and the remainder frozen at –20 °C. Each extraction included a negative control to check for contamination.

Museum samples

Typically, half of a saddleback toepad and an entire robin toepad were used to extract DNA. Toepad samples were finely chopped and DNA was extracted with the Qiagen DNEasy kit. Samples were lysed overnight at 55 °C in 180 μ L of Buffer ATL and 20 μ L of proteinase K. Afterwards, 200 μ L of Buffer AL was added and the mixture was incubated at 70 °C

for 10 min. Following incubation, 200 μL of ethanol ($\geq 99.8\%$) was added, the mixture was centrifuged through a spin column at 6800 g for 1 min, and the flow-through was discarded. The DNA, which was then bound to the silica gel membrane in the spin column, was washed with 500 μL of Buffer AW1 and 500 μL of Buffer AW2 to remove contaminants and inhibitors. Finally, DNA was eluted twice with 200 μL of Buffer AE, giving two 200- μL volumes of extracted DNA. A working stock of DNA was stored at 4 °C and the remainder frozen at -30 °C. All analyses used DNA from the first elution.

Contamination is frequently a problem with old samples (Pääbo *et al.*, 2004), so extensive measures were taken to ensure that our results were genuine. Extractions and PCR set-ups occurred in a separate laboratory where no amplified or contemporary robin or saddleback DNA had ever been present and no laboratory materials or clothing were moved from the PCR/gel laboratory to the extraction/set-up laboratory. The extractions and PCR set-ups took place in a UV hood, which was irradiated with UV light to destroy DNA following each laboratory session. New pipettes were bought for these analyses, and filter tips and double-processed tissue culture water were always used. Every extraction and PCR had a negative control and approximately one-quarter of the samples were chosen randomly, and extracted and amplified twice ($n = 11$ for both species). Some authors suggest extensive replication for reliable genotyping, citing DNA concentrations in picogram amounts as a problem for historic samples (Taberlet *et al.*, 1996). We used a Nanodrop to determine the amount and purity (pure DNA has an absorbency ratio of ~ 1.8 in the 260/280 spectrum; Nano Drop Technologies Inc., 2006) of DNA yielded to assess whether such replication was necessary (Pääbo *et al.*, 2004).

Microsatellite analysis

Variation at polymorphic microsatellite loci was investigated using denaturing and nondenaturing gels (Table 2). For denaturing gels, 10 pmol of reverse primers were radioactively end-labelled in 10 μL of reaction volumes containing 5 μCi of [γ - ^{33}P -ATP], 2.5 units of T4 polynucleotide kinase (Bioline, London, UK), and 1x kinase buffer (Bioline). This mixture was incubated at 37 °C for 1 h and then heated at 90 °C for 2 min to denature the kinase.

A polymerase chain reaction was carried out in 10- μL volumes using 0.6 units of AB Gene Red Hot DNA polymerase, 12.5 nM radioactively labelled reverse primer, 0.45 μM unlabeled reverse primer, 0.5 μM forward primer, 0.8 mM dNTPs and 1x buffer (AB Gene). Amplification conditions were optimized for each locus (Table 2) by varying the annealing temperature, concentration of MgCl_2 and DNA and use of betaine (1.1 M final

concentration) and dimethylsulfoxide (2% v/v). Typically, the PCR profile consisted of denaturation at 92 °C for 3 min, followed by 35 cycles at the annealing temperature for 30 s, 72 °C for 1 min, and 92 °C for 1 min followed by one final annealing step for 30 s and extension at 72 °C for 4 min.

After PCR, 4 μL of formamide loading dye was added to each sample and 6 μL of the mixture was electrophoresed on a 6% polyacrylamide gel for 3–4 h. Gels were dried for 2 h and exposed to autoradiograph film to visualize alleles. Individuals expressing all known alleles were run on every gel as size standards.

The protocol for nondenaturing gels differed in some respects. For the PCR reaction, 0.5 units of AB Gene Red Hot DNA polymerase, 0.5 μM forward and reverse primer, and bromophenol blue/cyanol/sucrose for the loading dye were used. PCR mixture (1–4 μL) was electrophoresed on 6–10% polyacrylamide gels (Table 2). Gels were run for 17–24 h, stained with 4 μL of SYBR green and photographed under UV light. Molecular rulers (10 or 20 bp ladders) were used as additional size standards.

Data analysis

Prior to statistical analyses, MicroChecker (Van Oosterhout *et al.*, 2004) was used to determine the likelihood of large allele dropout, null alleles and scoring error due to stuttering. Genepop version 3.4 (Raymond & Rousset, 1995) was then used to check for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (Fisher's exact tests) using 10 000 dememorizations, 1000 batches and 10 000 iterations. Where appropriate, sequential adjustment of critical P -values using Bonferroni correction was implemented (Rice, 1988). The critical value used in all tests was $\alpha = 0.05$.

For saddlebacks, Kaimohu Island, Big Island, Big South Cape Island and all mainland museum samples were treated as four separate populations. The mainland museum samples were checked for genetic structure by testing for genic differentiation among three areas with the largest sample sizes (Fiordland, Taramakau and Otago) using Genepop v. 3.4. To ensure that heterozygosity in mainland historical samples reflected additional alleles throughout the mainland rather than highly structured mainland populations with individually low levels of heterozygosity, we calculated and graphed H_O for each mainland individual and compared the Big South Cape, Big and Kaimohu populations. We also plotted allele frequencies at the six loci polymorphic in contemporary island samples as well as historic Fiordland, Taramakau, Otago and Big South Cape Island samples.

Robin museum samples came from several locations, but sample sizes from individual locations were too small to perform meaningful statistical analyses. Instead, we placed samples into two groups: island (excluding Stew-

Table 2 Optimized PCR conditions used to amplify saddleback and robin microsatellite loci.

Locus	Primer sequence (5'–3')	Allele size (bp)	Gel type	[MgCl ₂] (mM)	Anneal (°C)	Gel	Betaine/DMSO
Saddlebacks							
2F9*	f GCATTCTGGGCTGTAACAT r AAAGGACAATGTAATTGGTG	100–114	Nondenaturing	1.5	55	9	N
Ase18	Richardson <i>et al.</i> (2000)	216–222	Denaturing	1.5	56	6	N
CK5A4B	Tarr & Fleischer (1998)	124–126	Nondenaturing	2	52	10	Y
Hr μ 6	Primmer <i>et al.</i> (1996)	201–287	Denaturing	1.5	56	6	N
K3/4	Hudson <i>et al.</i> (2000)	117–125	Nondenaturing	1.5	58	9	N
K9/10	Hudson <i>et al.</i> (2000)	75–97	Nondenaturing	2.0	54	9	Y
K13/14	Hudson (1999)	242–278	Nondenaturing	1.5	58	6	N
K15/16	Hudson (1999)	92–118	Nondenaturing	2.0	58	9	Y
Pca01	Lambert <i>et al.</i> (2005)	178–192	Nondenaturing	1.5	57	7	N
Pca02	Lambert <i>et al.</i> (2005)	109–111	Nondenaturing	1.0	57	9	N
Pca05	Lambert <i>et al.</i> (2005)	138–160	Nondenaturing	0.8	58	9	N
Pca07†	f GGCCAGACTCAGTCTGAACG r Lambert <i>et al.</i> (2005)	130–136	Nondenaturing	1.5	57	9	N
Pca08	Lambert <i>et al.</i> (2005)	82–84	Nondenaturing	1.5	55	9	N
Pca12	Lambert <i>et al.</i> (2005)	106–116	Nondenaturing	1.5	55	9	N
Pca13	Lambert <i>et al.</i> (2005)	158–162	Nondenaturing	1.5	55	8	N
Pca14	Lambert <i>et al.</i> (2005)	106–110	Nondenaturing	1.5	55	9	N
Pca15	Lambert <i>et al.</i> (2005)	201–203	Denaturing	1.5	63	6	N
Pcc2‡	f GATCCGGACTGCTGGGC r TGCCGTCTGGGTGCGTG	88–96	Nondenaturing	1.0	57	9	Y
Pcc4‡	f ATCTCCACAAGAGCAGGA r TCAAACCACTACACCAGG	82–102	Nondenaturing	1.5	50	9	N
Pgm1	Dowling <i>et al.</i> (2003)	190–214	Nondenaturing	2	54	7	N
Pocc 1	Bensch <i>et al.</i> (1997)	107–115	Nondenaturing	1.5	56	9	N
Pocc 6	Bensch <i>et al.</i> (1997)	174–176	Nondenaturing	2.5	52	7	N
Pocc 8	Bensch <i>et al.</i> (1997)	215–219	Denaturing	2.5	50	6	N
Robins							
2F9*	f GCATTCTGGGCTGTAACAT r AAAGGACAATGTAATTGGTG	84–102	Nondenaturing	1.5	57	9	Y
4E8*	f TGTGGGAAACCAGAGGAAA r CAGGGGAAAAATAGAGAGGG	96–108	Nondenaturing	1.5	56	9	N
Ase18	Richardson <i>et al.</i> (2000)	170–174	Nondenaturing	1.5	56	7	N
Ase64	Richardson <i>et al.</i> (2000)	290–316	Denaturing	1.5	57	6	N
Esc μ 6	Hanotte <i>et al.</i> (1994)	110–120	Nondenaturing	1.5	56	9	N
GgaM μ 128	Gibbs <i>et al.</i> (1997)	174–176	Denaturing	1.5	57	6	N
Indigo 28	Sefc <i>et al.</i> (2001)	163–172	Nondenaturing	2	50	8	N
Pca13	Lambert <i>et al.</i> (2005)	156–160	Nondenaturing	1.5	56	8	N
Pgm3	Dowling <i>et al.</i> (2003)	170–174	Nondenaturing	2.5	52	7	N
Pocc6	Bensch <i>et al.</i> (1997)	180–186	Denaturing	1.5	60	6	N

*Unpublished primer sequence from Lambert *et al.* (2005), T. King, personal communication.

†Forward primer redesigned from Lambert *et al.* (2005).

‡Unpublished primer sequence, T. King, personal communication.

art Island, $n = 8$) and mainland (including Stewart Island, $n = 32$). Similarly, we combined contemporary robin data for Nukuwaiata ($n = 25$) and Breaksea ($n = 55$) Islands to create an island sample ($n = 80$) and combined data for the Eglinton Valley ($n = 170$), Flagstaff Point ($n = 12$) and Nelson Lakes ($n = 21$) to produce a mainland sample ($n = 203$) for comparisons with museum samples.

Genetic diversity within each population was quantified using the number of polymorphic loci, number of alleles and the mean number of alleles per locus. To adjust for sample size in the number of alleles per locus,

allelic richness was calculated in Fstat (Goudet, 1995). Heterozygosity was given as mean observed heterozygosity (H_O) and mean expected heterozygosity (H_E). Among the four saddleback populations noted above, differences in the mean number of alleles per locus, allelic richness and H_E were tested using Wilcoxon signed-rank tests. We also used these variables to test for temporal and island/mainland differences between museum and contemporary robin samples.

We tested Kaimohu, Big and Big South Cape Island saddleback populations for bottlenecks using the programs BOTTLENECK v. 1.2.02 (Piry *et al.*, 1999) and Garza

& Williamson's M (2001). In BOTTLENECK, we used all three mutation models: the infinite allele model (IAM), the step-wise mutation model (SMM) and the two-phase model of mutation (TPM). We examined results for two statistical tests appropriate for populations with < 20 polymorphic loci (sign and Wilcoxon signed-ranks tests) and allele frequency distributions (mode-shift indicator) to identify bottlenecked populations for each mutation model (Luikart *et al.*, 1998). Garza & Williamson's M (2001) detects bottlenecks within the last 135–500 generations using the mean ratio of the total number of alleles to the range in allele size, a ratio that is smaller in recently reduced populations. Assuming that the Big South Cape Island population was founded by mainland birds, we calculated M using $\mu = 5 \times 10^{-4}$ (Garza & Williamson, 2001) and two values (10 and 20) for θ ($4N_e\mu$), which correspond to equilibrium effective population sizes on the mainland of 5000 and 10 000 birds. The effective population size of the mainland population is unknown, but increasing the effective population size reduces the probability of finding a significant result if all other parameters are kept equal. If 5000 and 10 000 individuals do not produce significant results, then larger populations will also be nonsignificant. For Kaimohu and Big Islands, which were founded by Big South Cape Island birds, we used three values for θ (1, 2 and 4) to calculate M , which correspond to effective population sizes of 500, 1000 and 2000 birds. Big South Cape Island is 911 ha (Merton, 1975) and probably would not have supported more than $N_e = 2000$ (Hoo-son & Jamieson, 2003). Kaimohu and Big have known bottlenecks, which should be identified by both programs, but, because the programs detect relatively recent bottlenecks, we do not expect to see evidence for a bottleneck in the Big South Cape Island population if it was ancient and associated with a founder effect during colonization of the Island.

Results

DNA quantification and microsatellite loci

Museum saddleback DNA had a mean purity (A_{260}/A_{280}) of 1.68 ± 0.14 (range 1.31–1.86) and a mean concentration of 21.02 ± 13.75 ng/ μ L (range 5.95–56.30 ng/ μ L). Mean robin sample purity was 1.58 ± 0.18 (range 1.05–1.84) and contained a mean DNA concentration of 17.67 ± 11.03 ng/ μ L (range 3.25–55.00 ng/ μ L). These results indicate high DNA quality and yield for both species (Taberlet *et al.*, 1996; Nano Drop Technologies Inc., 2006).

Three robin museum samples (Appendix 1b) did not amplify for most loci and were excluded, giving a final sample size of 40. Two robin loci and one saddleback locus showed potential problems with amplification and null alleles. The two loci with the largest alleles (robin Ase 64 and saddleback K13/14) only amplified for 16/43

and 13/44 museum skins respectively. We believe that these products were too large (290–316 and 242–278 bp) to be amplified reliably across all samples using degraded museum DNA (Pääbo *et al.*, 2004). One robin locus (Pca12) showed homozygote excess in museum samples and did not amplify consistently between replicate museum extractions. We excluded these three loci from our analyses.

No other locus showed evidence of large allele drop-outs, null alleles or scoring error due to stuttering in any population (saddleback mainland museum samples had insufficient sample size to run MicroChecker). All analysed loci gave identical results for replicate extractions and PCR reactions. None of the negative controls showed contamination.

Genotypic distribution and linkage disequilibrium

For saddlebacks, there were no significant departures from HWE for any locus in the Kaimohu, Big and Big South Cape Island populations, but the mainland museum samples showed a significant heterozygote deficit at 6/22 loci ($P < 0.05$). For robin samples, all loci were consistent with HWE ($P > 0.05$). Saddleback mainland museum samples were obtained from several different locations. If mainland populations were genetically structured, this could lead to heterozygote deficit when samples were pooled (Wahlund effect). In support of this idea, there was significant genic differentiation among the Fiordland, Taramakau and Otago populations ($\chi^2_{44} = 74.12$, $P = 0.003$). No linkage disequilibrium was detected for saddlebacks or robins between any locus pair across all populations ($P > 0.05$).

Allelic diversity

Contemporary and historic saddleback populations

Mainland saddleback museum samples had 22 polymorphic loci, but only six of these were polymorphic for historic Big South Cape samples and contemporary Big Island samples. Five of these were polymorphic for contemporary Kaimohu Island samples (Table 3). All loci polymorphic in historic Big South Cape and contemporary populations were polymorphic in historic mainland samples. At least 75% of historic mainland alleles were absent both in historic Big South Cape and contemporary Big and Kaimohu samples (Table 3). Mainland and the three island populations of saddlebacks showed highly significant differences for the number of alleles per locus, allelic richness and expected heterozygosity (Table 3). In contrast, there were no significant differences for any of these measures among historic Big South Cape and contemporary Big and Kaimohu Island populations (Table 3).

To ensure that the high genetic variation observed in historic mainland samples was not solely the result of population subdivision, we compared individual H_O of

Table 3 Allelic diversity and mean heterozygosity in contemporary and historic saddleback and robin populations.

Species	Source	<i>n</i>	Polymorphic loci	No. of alleles	Alleles per locus	Allelic richness	H_O	H_E
Saddlebacks								
Museum	Mainland	24	22	143	6.50 abc	5.71 abc	0.3903	0.5323 abc
	Big South Cape Island	20	6	35	1.59 a	1.56 a	0.1566	0.1369 a
Contemporary	Kaimohu Island	16	5	31	1.41 b	1.40 b	0.1054	0.1000 b
	Big Island	35	6	32	1.45 c	1.45 c	0.1263	0.1278 c
Robins								
Museum	Mainland samples	32	8	30	3.75 a	2.77 a	0.3715	0.3899 a
	Island samples	8	6	19	2.38 a	2.38	0.3750	0.3905
Contemporary	Mainland samples	203	8	29	3.63 b	2.59 a	0.3235	0.3428 a
	Island samples	80	6	21	2.63 b	2.39	0.3203	0.3846

To test for differences in genetic variation, pair-wise comparisons of alleles per locus, allelic richness and H_E were carried out with Wilcoxon signed-rank tests. For robins, museum and contemporary samples were subdivided into island and mainland sites to control for potential differences (there are no contemporary mainland populations of saddlebacks). Similar letters in columns join significantly different pairs; for saddlebacks, all P -values were significant at $P < 0.0001$, nonsignificant values ranged between 0.18 and 0.60. For robins, significant P -values ranged between 0.023 and 0.050, and nonsignificant values ranged between 0.161 and 0.917.

mainland birds to individual H_O of Big South Cape, Big and Kaimohu birds (Appendix 2). All but one of 24 museum specimens had higher H_O than all 71 birds from Big South Cape and the two contemporary populations (Appendix 2). Furthermore, allele frequencies for the six loci polymorphic in the contemporary samples showed much higher diversity in birds collected from each of the three historic areas on the mainland, with numerous additional alleles compared with contemporary specimens (Appendix 3). These results demonstrate that greater diversity on the mainland is not the result of highly structured but individually invariant populations, rather all mainland individuals and populations show high levels of diversity.

BOTTLENECK showed no evidence for a bottleneck event in the Kaimohu Island population ($P > 0.156$), all tests were significant for the Big Island population ($P < 0.019$), and one test was significant for the Big South Cape population (Wilcoxon test under the IAM mutation model; $P = 0.031$). Garza & Williamson's M (2001) was higher than the 0.68 critical value (bottlenecked populations are indicated by values ≤ 0.68), suggesting that bottlenecks have not occurred in any of the three populations (Kaimohu 0.86; Big 0.78; Big South Cape 0.83).

Contemporary and historic robin populations

Robins appear to have lost some genetic variation over time, but show few differences between island and mainland sites. Two alleles present in museum samples were not observed in contemporary birds and one allele present in contemporary birds was not observed in museum samples. For mainland samples, differences between museum and contemporary samples were small but significant for allelic richness (2.77 museum vs. 2.59 contemporary) and expected heterozygosity (0.39 museum vs. 0.34 contemporary; Table 3). For island

samples, there was no significant difference between museum and contemporary samples for the number of alleles, allelic richness or expected heterozygosity (Table 3). Overall, mainland robin populations had more polymorphic loci and significantly more alleles per locus than island populations both within the contemporary and museum samples. However, there were no significant differences in allelic richness (which corrects for sample size; El Mousadik & Petit, 1996) or expected heterozygosity (Table 3).

Discussion

Accuracy of the results from historic samples

We believe that the results we obtained for the museum samples are very reliable. There was no sign of contamination, DNA concentrations were in nanogram amounts (1000x more concentrated than the picogram amounts discussed by Taberlet *et al.*, 1996) and of good quality (Nano Drop Technologies Inc., 2006), loci with large allele sizes tended not to amplify as expected for degraded museum DNA, and our replicates produced identical results (Pääbo *et al.*, 2004). Finally, we found numerous novel alleles in the historic saddleback samples that were not present in contemporary samples from Big and Kaimohu Islands or in four other contemporary saddleback populations ($n = 453$) genotyped in a concurrent study (Taylor *et al.*, unpublished manuscript).

Temporal changes in genetic diversity

Different bottleneck histories and ability to disperse and tolerate introduced predators have profoundly affected current genetic variation in saddlebacks and robins. The extinction of South Island saddlebacks on the mainland resulted in the loss of substantial genetic diversity.

At least 108 of 143 alleles (75.5%) present in the historic mainland samples were not detected in the historic remnant population on Big South Cape Island, and all other measures of genetic diversity show a similar pattern (Table 3). Furthermore, there is no significant difference between the two translocated populations on Kaimohu and Big Islands (contemporary samples) and their source, Big South Cape Island (historic samples; Table 3). These results suggest that the low genetic variation observed in contemporary South Island saddleback populations is a consequence of past bottleneck(s) or historic drift and not the recent decline caused by the rat plague in 1964, or from founder effects and subsequent drift as the translocated populations were established. Although future population declines may threaten saddlebacks for stochastic demographic reasons, they are unlikely to cause further substantial losses of genetic diversity in what is already a genetically depauperate species.

The distribution of South Island robins has also been negatively affected by introduced predators, but not to the same extent as saddlebacks, as robins continue to exist in isolated habitat patches on the mainland. Historic mainland samples of robins had slightly more genetic diversity than contemporary mainland samples, although historic island samples appeared to have similar levels of genetic variation compared with contemporary island samples (Table 3). The small temporal loss of genetic variation on the mainland may be attributable to habitat fragmentation and subsequent drift in disjunct robin populations because the three mainland populations are geographically distant. Loss of genetic variation may be greater than our results indicate if the loss of other rare alleles went undetected due to the small sample size for museum specimens (Sjögren & Wyöni, 1994).

Island robin populations appeared to have similar levels of genetic variation compared with mainland populations both within the historic and contemporary samples. All of the robin island populations we studied (Nukuwaiata, Motuara and Breaksea Islands) are adjacent to large mainland populations of robins (northern South Island and Fiordland). If robins can disperse from mainland populations to nearby islands, but are unable to reach other more distant mainland populations, then loss of genetic variation may occur for fragmented populations on the mainland but not between adjacent mainland and island sites.

In saddlebacks, the loss of allelic diversity and rare alleles in historic samples from Big South Cape Island compared with the mainland indicate a past bottleneck, possibly caused by a founder event during colonization of the island, and/or a long period of genetic drift. We expected *BOTTLENECK* and *M*, which detect relatively recent bottlenecks, to show a bottleneck event on Big and Kaimohu, but not on Big South Cape Island assuming Big South Cape was bottlenecked several thousand years before present. However, neither program detected the known bottleneck on Kaimohu Island,

which represented a population decline of 1000 to 16 birds. *BOTTLENECK* may have failed because Kaimohu has one less polymorphic locus than the other two populations and *BOTTLENECK* does not consider monomorphic loci, a limitation for loci that are polymorphic in some populations, but monomorphic in others as a result of bottlenecks (Williams *et al.*, 2002). The *M* ratio may not have produced a significant result because it does not perform well for changes from small populations in which drift is important (e.g. Big South Cape Island), to populations that are bottlenecked to even smaller sizes (e.g. Kaimohu and Big Islands; Garza & Williamson, 2001). Neither program systematically detected a bottleneck on Big South Cape Island, which is consistent with our prediction of an ancient founding event. Big South Cape Island may have been colonized during a glacial period when the continental shelf was exposed and land was continuous among Big South Cape Island, the South Island and Stewart Island (c. 169 000 ha; New Zealand's largest island after the North and South Islands). Sea levels rose when the last interglacial period began 10 000–15 000 years ago, and separated Big South Cape Island from Stewart Island by approximately 2 km of open water (Fig. 1; McGlone *et al.*, 2003). Any population die-off/recovery on Big South Cape Island during an inter-glacial period when gene flow with the mainland was limited or nonexistent would produce the low genetic variation seen in the Big South Cape Island saddleback museum samples. Similarly, drift occurring over 1200 generations (or 9600 years) would produce the ratio of H_E values [$(H_{t+1}/H_t) = 0.3$] observed between a mainland population (Taramakau) and Big South Cape, assuming an effective population size of 500 individuals on Big South Cape Island (using $t = -2N_e \cdot \ln(H_{t+1}/H_t)$).

The low historic genetic variation of saddlebacks on Big South Cape Island and some minor loss of diversity following the translocations to Big and Kaimohu Islands have resulted in two contemporary populations with very low expected heterozygosity (0.10 and 0.13). These measures are similar to Mauritius and Seychelles kestrels (0.10 and 0.12), which were bottlenecked to very small population sizes; a single breeding pair for the Mauritius kestrel (Groombridge *et al.*, 2000). Low levels of genetic diversity can reduce a population's ability to evolve new adaptations and reduce resistance to disease and parasites thereby increasing the probability of extinction (Newman & Pilson, 1997; Saccheri *et al.*, 1998; Coltman *et al.*, 1999; Hughes & Boomsma, 2004). Consequently, the low levels of genetic variation observed in remaining saddlebacks may be a cause for concern.

However, saddlebacks have likely persisted in isolation for thousands of years on Big South Cape Island with extremely low genetic variation. Such long-term persistence coupled with low genetic variation has been demonstrated for an endemic South American mammal, the tuco-tuco (*Ctenomys sociabilis*), with individuals showing virtually identical cytochrome *b* sequences over

the course of 1000 years (Hadly *et al.*, 2003). These examples and others (Hitchings & Beebe, 1996; Visscher *et al.*, 2001) question the view that low genetic variation necessarily threatens fitness and population persistence. Small populations may maintain fitness through purging (Visscher *et al.*, 2001; but see Hedrick, 1994; Crnokrak & Roff, 1999), beneficial mutations and sexual selection (Whitlock, 2000), back-mutations of deleterious alleles to the original functional alleles (Lande, 1998) and new mutations that compensate for the negative fitness effects of fixed deleterious alleles (Burch & Chao, 1999; Whitlock & Otto, 1999). These explanations may singly or in combination account for persistence of stable populations with low genetic variation in some species. The relationship between fitness and genetic variation at neutral markers is, however, unclear (Lehman, 1998), so studies showing long-term genetic invariance do not necessarily contradict studies that use replicate populations and show decreased survival for those with low genetic variation (Saccheri *et al.*, 1998). Until the function and effectiveness of mechanisms that may maintain fitness are confirmed, it would be prudent to protect genetic variation in species, such as saddlebacks, that may have little resilience to rapid and human-driven environmental changes.

Dispersal and genetic variation in island and mainland populations of threatened species

Conservation managers frequently rely on isolated areas, particularly offshore islands, to protect endemic species from extinction by introduced predators (Eldridge *et al.*, 2004). Although island refuges provide excellent protection, our results suggest that species with poor dispersal capability that have naturally persisted on islands may have low genetic variation as a result of past drift, founder events or bottlenecks. Few studies have explicitly examined differences in genetic variation between mainland and island populations of threatened species, but those that have show reduced genetic variation for the island populations (Eldridge *et al.*, 2004; Jamieson *et al.*, 2006).

The importance of dispersal capability and island/mainland sites for the maintenance of genetic variation is illustrated by the different results obtained for saddlebacks and robins. By the early 1900s, genetically diverse saddlebacks from the mainland were virtually extinct. The saddlebacks that remained were confined to one small, historically bottlenecked island, which has resulted in a permanent loss of allelic diversity in the species. Had saddlebacks on the mainland passed through a bottleneck of similar size to the rat bottleneck on Big South Cape Island and survived, they would have certainly retained more genetic variation than that observed on Kaimohu and Big Islands. Each of the three historic mainland subpopulations (Taramakau, Otago and Fiordland) with sample sizes of just five to six birds showed far more

genetic variation than the entire sample ($n = 20$) from the Big South Cape group (Appendix 3). Furthermore, individuals from the mainland typically had higher levels of H_O than individuals from Big South Cape or contemporary populations (Appendix 2). Retaining allelic diversity may be crucial to evolutionary potential because single alleles are often important for disease and insecticide resistance (Allendorf, 1986; Fuerst & Maruyama, 1986; McKenzie & Batterham, 1994). Therefore, every effort should be made to protect diverse mainland populations particularly for species that do not readily disperse.

In contrast to saddlebacks, contemporary robins were never extirpated from the mainland by introduced predators and are capable of longer sustained flights than saddlebacks, mitigating founder effects on islands and promoting gene flow among populations. Consequently, robins appear to have lost very little genetic variation since historical times. Although some contemporary robin populations have individually lost alleles and show reduced heterozygosity (Boessenkool *et al.*, 2006), the loss is not permanent because managers could potentially reintroduce missing alleles and increase heterozygosity through translocations among the remaining populations if deemed necessary.

Dispersal capability in robins has also meant that wholesale extirpation on the mainland would not have caused the serious loss of genetic variation observed for saddlebacks as considerable genetic variation would have been preserved. Although genetic variation in mainland samples tended to be higher than island samples, this difference was not significant for any measure of genetic variation except the number of alleles per locus, which was slightly higher (one extra allele per locus) for mainland samples (Table 3). Dispersal capability of robins had probably increased historic gene flow among populations and produced island populations with most of the genetic variation observed on the mainland.

Inferring loss of genetic variation following population declines

Recent population declines are inferred to be the cause of low contemporary patterns of genetic variation in several studies that lack information on prebottleneck levels of genetic variation (e.g. O'Brien & Evermann, 1988; Snowbank & Krajewski, 1995; Houlden *et al.*, 1996; Hoelzel, 1999). This assumption has a strong theoretical basis and is also substantiated by several studies that have used historic DNA to confirm higher genetic variation for prebottlenecked compared with post-bottlenecked populations (Bouzat *et al.*, 1998; Glenn *et al.*, 1999; Groombridge *et al.*, 2000; Whitehouse & Harley, 2001; Larson *et al.*, 2002). However, attributing low current genetic variation to recent population bottlenecks can be erroneous. Increasingly, studies show that low levels of

contemporary genetic variation are the result of ancient and not recent bottlenecks (Ellegren *et al.*, 1996; Culver *et al.*, 2000; Matocq & Villablanca, 2001; Pertoldi *et al.*, 2001; Paxinos *et al.*, 2002). For example, nene (*Branta sandvicensis*) showed a prehistoric loss of genetic variation that coincided with a period of prehistoric human population growth (Paxinos *et al.*, 2002). Likewise, similar levels of genetic variation for contemporary and museum DNA in European otters (*Lutra lutra*) was attributed to post-glacial founder events or historic declines associated with prehistoric human activity rather than recent population declines (Pertoldi *et al.*, 2001). If low genetic variation can be characteristic of large mainland populations as a result of post-glacial re-colonization or prehistoric human activity, is it also characteristic of isolated populations historically established with few founders? In South Island saddlebacks, we showed that the loss of genetic variation on Big South Cape Island is not the result of a population decline from ~1000 to 36 birds caused by the rat plague in 1964, but most probably the result of long-term drift, an ancient bottleneck or natural founding event to the island. Arguably, many species reduced from large mainland populations to small, relict island populations may have exceptionally low genetic variation as a consequence of historical events, especially species with poor mobility. In New Zealand alone, North Island saddlebacks, the flightless takahe (*Porphyrio mantelli*) and kakapo (*Strigops habroptilus*), two frog, two tuatara and six lizard species were typically widespread on the mainland, but are now only present on small islands and/or isolated montane areas, a situation that has potentially exacerbated loss of genetic variation and contributed significantly to the very low genetic variation now observed in these species (Townes & Daugherty, 1994; Bell *et al.*, 1998; Holyoake *et al.*, 2001; Hay *et al.*, 2003; Miller *et al.*, 2003; Lambert *et al.*, 2005; Jamieson *et al.*, 2006).

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Supplementary material

The following supplementary material is available for this article:

Appendix S1 Location, date and museum accession numbers for historic saddleback and robin samples.

Appendix S2 Observed heterozygosity for each individual saddleback in every population: Mainland, Big South Cape, Big and Kaimohu.

Appendix S3 Allele frequencies for six loci that were polymorphic in contemporary saddlebacks from Kaimohu and Big Islands compared with allele frequencies for the same six loci in historic Big South Cape Island and three historic mainland areas.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1420-9101.2007.01362x>

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