The spatial ecology and phylogeography of the grand skink (*Oligosoma grande*): implications for the conservation of an endangered species

Oliver Fleetwood Berry

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"The matrix matters"
Ricketts, 2001

Overleaf. A view of grand ridge looking northeast along the boundary between pasture (left) and tussock grassland (right). The large rock rising above the pasture to the far left of the photograph is study rock P1.1, and is referred to in chapters 3, 4, and 5. Study site T2 is located in tussock grassland to the immediate right of the centre of the image.

The spatial ecology and phylogeography of the grand skink (*Oligosoma grande*): implications for the conservation of an endangered species

Abstract

Species conservation relies on determining the mechanisms that cause endangerment. This thesis documents the conservation ecology and phylogeography of an endangered lizard, *Oligosoma grande*, from southern New Zealand, and tests hypotheses that may explain its endangered status.

Grand skinks are large rock-dwelling lizards endemic to montane grasslands in central Otago, New Zealand. They are classified as vulnerable to extinction by IUCN criteria and are the subject of intensive management. The causes of their endangerment are not well understood, but changes to landscape connectivity caused by agricultural development have been implicated. I developed microsatellite and mitochondrial DNA markers, and combined them with field studies to address four key problems relevant to the conservation of this species. First, I investigated the ability of newly developed analytical tools for genetic data (assignment tests) to provide qualitative and quantitative dispersal information. I show that assignment tests can provide highly accurate dispersal information, and describe the circumstances where they perform best. Second, building on these results, I combined genetic and demographic data to provide an empirical account of how two forms of agricultural landuse affect the connectivity of skink populations. I show that the nature of the vegetation matrix between skink populations both quantitatively and qualitatively affected dispersal patterns, but also that characteristics of individual populations play a role in determining dispersal dynamics. Third, following the evidence that grand skink populations were very insular, I used parentage and kinship analysis to investigate the extent of inbreeding and inbreeding avoidance behaviours. It is often assumed that close inbreeding is harmful and animals should avoid it by either dispersing after birth, or recognising and avoiding mating with kin. However, I found no evidence for inbreeding avoidance or inbreeding depression. Finally, I used phylogeographic analyses to understand the contributions of historical and contemporary processes to *O. grande*'s rarity and fragmented distribution. I demonstrate that that the current fragmented distribution has both historical as well as recent anthropogenic origins. Some populations show evidence of recent declines that may have occurred since human settlement in New Zealand, but in addition, more ancient biogeographic processes have driven population fragmentation.

Acknowledgements

Although a thesis has a single author, many people contribute, and in many ways. To everyone that has answered my questions, asked me questions, disagreed, provided their unpublished data, drawn me a map, allowed me onto their property, pointed me in the right or wrong direction, written on the ecology of New Zealand, given me a lift, made me laugh, thankyou.

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Special thanks to my parents, Gill and Paddy for giving me all the opportunities that I have had, and along with Matt, for showing me the value of family. And to my infant son Huxley for showing me that there is more to life than microsatellites and skinks. Finally, my wife and constant companion Nicki Mitchell – for making me come home, for showing me how to live in the moment, propping me up when I needed it, and for being my inspiration.

Many others contributed to aspects of this thesis and are acknowledged in the relevant chapters.

1

Introduction

Thesis structure and scope

This thesis documents the conservation ecology and phylogeography of an endangered lizard, *Oligosoma grande*, from southern New Zealand, and tests hypotheses that may explain its endangered status. The thesis is structured as a series of five self-contained scientific manuscripts that serve as chapters. Here, I introduce the grand skink case study, and the circumstances that prompted this research.

Background to this thesis

The New Zealand biota possesses high endemism and many evolutionary novelties (Daugherty et al., 1993). Lizards (skinks and geckos) are a prominent component of the terrestrial vertebrate fauna, and on an area-specific basis, species diversity exceeds that of similar temperate regions elsewhere (Daugherty et al., 1994). Twenty-nine species of skink occur in New Zealand, and additional cryptic species are likely to be described following further molecular study. Extant species fall within two genera, *Oligosoma* and *Cyclodina*, and are members of the Eugongylus group of Indo-Pacific skinks (Hutchinson & Donnellan, 1993).

Like most of the New Zealand vertebrate fauna, and typical of island biotas worldwide (Milberg & Tyrberg, 1993; Atkinson & Cameron, 1993), nearly half the lizard species are threatened with extinction or are restricted to offshore islands, and many are managed for their conservation (Towns *et al.*, 2001; Towns *et al.*, 2002). Many declines can be attributed to humans and their commensal animals (e.g. Whitaker, 1978; Towns, 1985), but the precise mechanisms of decline are often not understood (Towns et al., 2001).

Grand skinks, *Oligosoma grande*, are among the largest lizards in New Zealand (to 111 mm snout-vent-length and 24 grams), and are endemic to central Otago, South

Island (Plate 1). They are rock dwelling, and typically live in small groups (*ca.* 10-20 individuals) on schist rock outcrops in montane tussock grasslands (Plate 2). Like almost all New Zealand lizards, grand skinks are viviparous (live-bearing), and females have a low annual reproductive rate (2.17 offspring/year, Cree, 1994). They are also slow to mature (in their third or fourth year, Cree, 1994; Whitaker, 1996), long lived (to > 12 years, M. Tocher pers. comm.), and omnivorous (Tocher, 2003).

Grand skinks are uncommon. The holotype was described in 1845 (Hardy, 1977), but few records of the species existed until systematic surveys were conducted in the 1980s (summarised in Whitaker & Loh, 1995). These surveys revealed that most populations were small, and that the species had a disjunct distribution composed of an eastern and western cluster of populations. Estimates of the total number of skinks range between 1800 and 5000 (Patterson, 1992; Whitaker & Loh, 1995).

There is serious concern for the long-term survival of *O. grande*. Their fragmented distribution has been interpreted as resulting from a recent widespread decline, and population extinctions have occurred within the past few decades (Whitaker & Loh, 1995). Grand skinks have the highest conservation priority of any mainland New Zealand reptile, and the IUCN red data book lists them as vulnerable to extinction, category C2a (Molloy & Davis, 1994; I.U.C.N., 2000). The species is the subject of a high level of management and monitoring (Whitaker & Loh, 1995; Whitaker & Houston, 2002), and has received much conservation-focused ecological research (e.g. Murphy, 1994; Cree, 1994; Whitaker, 1996; Coddington & Cree, 1997; Stanley, 1998; Marshall, 2000; Houghton, 2000; Tocher, 2003).

Identifying the mechanisms driving species endangerment is fundamental to successful conservation (Caughley, 1994), and at least seven potentially threatening processes have been identified for *O. grande* (Whitaker & Loh, 1995). However, none have been clearly linked to their decline. One of the best studied putative threats is habitat modification – in particular, replacement of native tussock grasslands with pasture. Livestock graze both tussock grassland and pasture, but tussock grasslands have been greatly reduced in area since European settlement (McGlone *et al.*, 1995; McGlone, 2001), and this reduction apparently coincided with the decline of *O. grande* (Whitaker & Loh, 1995).

Plate 1. The grand skink, *Oligosoma grande*, is a large rock-dwelling lizard that inhabits montane tussock grasslands in southern New Zealand.

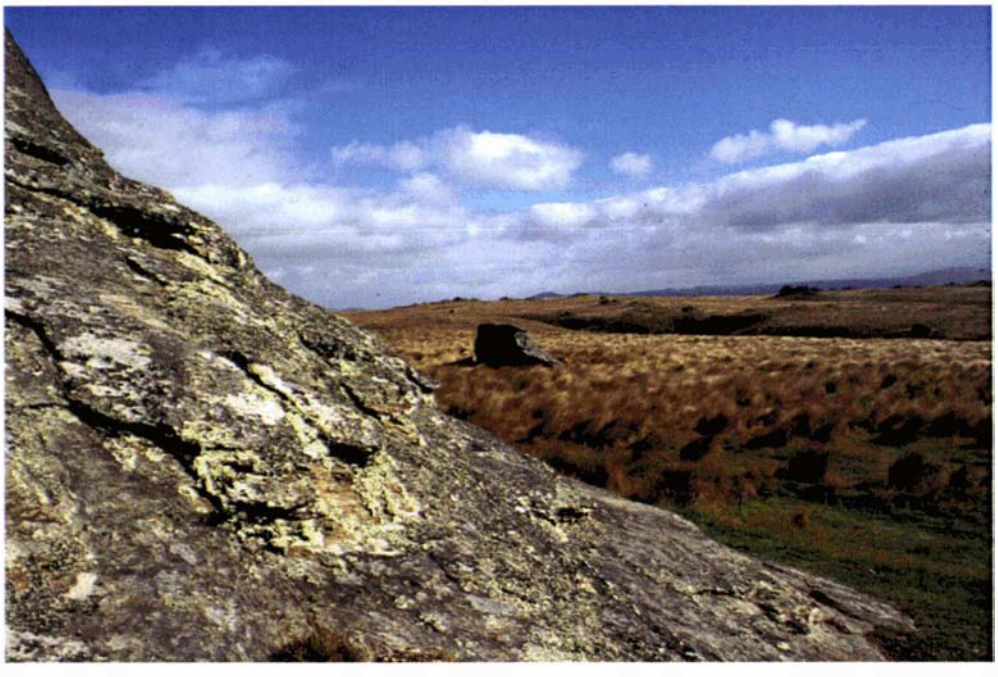

Plate 2. Montane tussock grassland in central Otago typically feature regularly spaced rock outcrops composed of highly fractured chloritic quartzo-feldspathic schist. Grand skinks are confined to these rock outcrops, usually in small groups of 10-20 individuals.

Evidence for a causal link between tussock grassland destruction and grand skink endangerment comes from a study by Whitaker (1996), who showed that grand skinks living on rock outcrops in pasture were less abundant, patchier, and more extinction-prone than those in tussock. He proposed that in natural situations grand skinks exist as a metapopulation, whereby recurrent dispersal maintains a balance between rock outcrop extinction and recolonisation (*sensu* Levins, 1968). Further, he suggested that pasture may inhibit dispersal between rock outcrops, resulting in a non-equilibrium metapopulation where recolonisations no longer balance extinctions (*sensu* Harrison, 1991), and a systematic decline in the occupation of rock outcrops follows (Whitaker, 1996).

The hypothesis that frequent dispersal allows grand skinks to persist in a patchy habitat has received little testing, primarily because of the difficulty associated with measuring long-term patterns of dispersal (see Koenig *et al.*, 1996). Consequently, a systematic examination of the spatial ecology of *O. grande* in both natural and modified landscapes is essential to their effective management. Addressing this issue forms the central aim of this thesis.

Approach

Although the objective of this thesis is to understand the ecology of a rare species, most inferences are derived from the analysis of microsatellite DNA markers and DNA sequences. As discussed in the chapters, analytical and technical advances in molecular research permit many questions to be addressed that were previously intractable by traditional ecological methods. Such molecular ecology is also a departure from the traditional role of genetics in conservation, which has been largely concerned with genetic variation *per se*, and has sometimes been controversial (Milligan *et al.*, 1994; Frankham, 2001; Elgar & Clode, 2001). This thesis investigates applied problems covering many levels of biological organisation, from the behaviour of individuals to their phylogenetic relationships. This is testament to the range of ecological information that DNA methodologies offer for the management of rare species.

Contributions of others to this thesis

The work contained in this thesis is entirely my own except where acknowledged. However, several of the chapters were published as co-authored papers, and it is appropriate that I comment on the contributions of the co-authors to this thesis. As is

common practice, my academic supervisors, Stephen Sarre and Dianne Gleeson, were co-authors in acknowledgement of their intellectual and physical contributions to the research. These contributions took the form of comments on preliminary project designs and draft manuscripts, and provision of funding and other resources. Mandy Tocher (Department of Conservation) was also co-author on manuscripts because she generously provided unpublished mark-recapture histories for the skinks that I studied. Aside from these contributions, I conceived the overall project and the objectives of individual chapters. I also developed the molecular markers, undertook fieldwork to collect tissue samples from the majority of the skinks, ran the genetic laboratory work, designed and executed the analysis, and wrote the manuscripts.

2

Microsatellite DNA markers for New Zealand skinks*

Reptiles are a prominent part of the faunal diversity of New Zealand, but most species have suffered major range declines or extinctions in the past 2000 years. Nearly fifty percent of species (26 of 62) are threatened or restricted to offshore islands and eight are subject to recovery programs (Towns & Daugherty, 1994; Towns et al., 2001). Most declines can be attributed to loss or modification of habitat and predation by introduced mammals (Towns *et al.*, 2001).

Previous genetic studies of New Zealand reptiles have increased the number of recognised species (e.g. Daugherty et al., 1990), but provided little autecological or population-level genetic information that could be applied to management. Here, I present the first microsatellite DNA markers to be developed for a New Zealand lizard. I used these markers to study dispersal in the grand skink, *Oligosoma grande*, a large rock-dwelling lizard endemic to sub-alpine grasslands in southern New Zealand (Chapters 3 and 4). This formerly widespread species now has a restricted range, and is listed as 'vulnerable' under IUCN criteria (I.U.C.N., 2000).

Tetranucleotide and dinucleotide microsatellite DNA was isolated from *O. grande* using an enrichment protocol modified from Armour et al. (1994). A detailed protocol is provided in Appendix I. Briefly, genomic DNA from 5 individuals was digested with *SAU* 3A, pooled, and the 300-600 base pair (bp) fraction ligated to SAU linkers (Royle et al., 1992). Fragments containing microsatellite DNA were selected by hybridisation to 3 mm^2 nylon membranes saturated with AAAG/TTTC, GATA/CTAT, GA/CT, or CA/GT target repeats. The enriched fraction was recovered by washing with SSC (150 mM sodium chloride, 15 mM sodium citrate) and 0.1% SDS (sodium dodecyl sulphate), then stripped from the membranes (100 µl 50mM KOH/0.01% SDS, followed

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by 100 µl 50 mM Tris HCl pH 7.5/0.01% SDS) and PCR amplified using SAU LA as the primer (95 °C, 5 min; 35 x (67°C 30 secs, 72°C 30 secs, 95°C 30 secs); 70°C 4 min).

SAU linkers were removed from the amplicons by digestion with *SAU* 3A. The DNA was purified (Highpure kit, Roche), ligated into pUC18 cut with *Bam* HI, and the plasmid transformed into Max Efficiency DH5α cells (Gibco BRL). Recombinant clones were identified by blue-white selection. Five hundred and fifty positive clones were cultured and screened for the presence of microsatellites by hybridisation to ^{32}P labelled $(AAAG)_n$, $(GATA)_n$, $(GA)_n$, or $(CA)_n$ concatemers. Twenty-eight percent of clones successfully hybridised to the probes.

After sequencing 70 positive clones using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, PCR primers were designed using OLIGO version 4.0 (National Biosciences). Loci were co-amplified in groups of three (see Henegariu *et al.*, 1997; Figure 1). Ten microlitre reactions consisted of 1 x PCR buffer (Roche), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µg/µL bovine serum albumin, 1 M Betaine, 0.5 U *Taq* polymerase (Roche), 20 ng skink genomic DNA, and primers. The PCR profile consisted of one cycle of 94°C for 2 minutes, 30 cycles of (94°C 30 secs, $T_{annealing}$ 20 secs, 72°C 30 secs), and a final extension at 72°C for 5 minutes. PCR was carried out on an iCycler thermal cycler (Biorad). Annealing temperatures and primer concentrations that gave the most even co-amplification are given in Table 1.

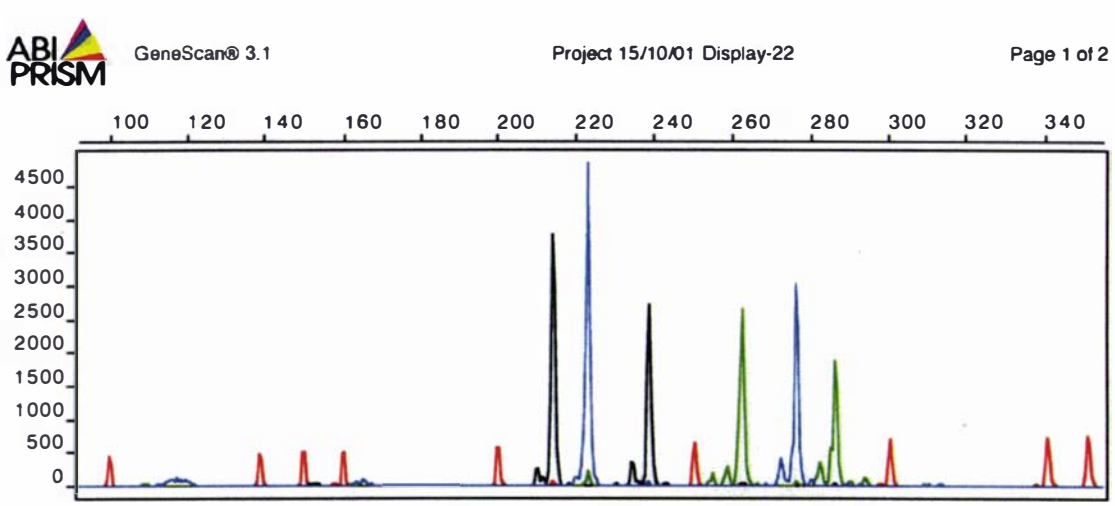

Figure 1. Genotype profile of three co-amplified tetranucleotide microsatellite loci (black peaks, locus Oligr4; blue peaks, locus Oligr3; green peaks, locus Oligr10). Red peaks are size standards, and numbers across the upper x-axis indicate size of alleles in base pairs. Note the small stutter bands, which are characteristic of tetranucleotide loci.

Table 1. Primers and PCR conditions used to amplify microsatellites in the grand skink, *Oligosoma grande*. Clone sequences are deposited in Genbank under accession numbers AF513228 - AF513242.

Locus	Primer sequence (5' - 3')	Repeat array	PCR multiplex set	Primer conc. (μM)	T_A °C	Size range of PCR product (bp)	Number of alleles	H_{obs}	H_{exp}	*n*
Oligr3	F CAGCCACCAGTATGTCAGTTG R TGCCTCTGCCACCAAATTAC	$GATA_{19}$	1	0.25	57	223 - 317	17	0.89	0.79	286
Oligr4	F ACCACCAAAAAGGCTCTGCTC R ACCCATTAGCCATTATTAGCC	$GATA_{15}$	1	0.30	57	197 - 258	15	0.80	0.77	288
Oligr10	F AGTGTGTTCTGACATTTGTGG R CCAGGATATACAGGATAACAG	$GATA_{18}(ATTT)GATT_9$	1	0.50	57	250 - 294	12	0.85	0.82	285
Oligr2	F GGAGAGTTCATTGTGTTCCTA R AGAACCACAACACCATCTGTA	$AAAG_{21}(GAAG)AAAG_2$	2	0.40	59	236 - 293	15	0.73	0.79	281
Oligr6	F TTTGGTGCCTTATTGCCTTTG R GGTCTTTGGGTCTATGCTTTG	$GATA_{18}$	2	0.50	59	126 - 220	18	0.81	0.80	287
Oligr13	F GGATCCCTTGTTCACCCCACA R CGTCCATCTGTCCATCTAATC	$GATA_{23}GATG_5$	2	0.30	59	200 - 326	26	0.77	0.80	285
Oligr7	F AAGCATCTTCGGTCACGAC R CAAGAAAATGTAGGGCACGAC	$GATA_{20}$	3	0.45	59	154 - 228	16	0.85	0.78	289
Oligr8	F CGGTCCAGCATCAGCCATCAC R GCACCACCCATTCAAAAGATGTG	$AAAG_{18}$	3	0.35	59	232 - 296	15	0.75	0.73	289
Oligr11	F CTTGAAGAGCAACGATGTCCA R GTTACTGATTGGCTGGGTGAC	$GATA_{22}$	3	0.40	59	179 - 264	15	0.84	0.79	289
Oligr1	F TGTCATTTCCCCCGTACTGTG R CCACCTCCCTCGGCATCATTC	$AAAG_{28}$	4	0.35	52.5	168 - 219	15	0.80	0.81	246
Oligr14	F TCTGGTTAACAGAGATTCCAC R AGACAGTGGTGAAGTTTGAAG	$GT_{20}(GATT)GT_3$	4	0.35	52.5	266 - 290	8	0.39	0.36	248
Oligr17	F GATCTGCAACCTATATGTACA R AGCAACCAAGAATACAAACAC	$CA_{13}(CG)CA_4$	4	0.45	52.5	264 - 305	13	0.82	0.80	248
Oligr15	F ACCCTACAACCAGTCCACTTC R CTGGGCACTGAGTTGGTATGA	CA_{25}	5	0.30	52.5	95 - 150	22	0.56	0.80	228
Oligr19	F CTGTCTGCTGCTAATGGAGAG R AAACACCCCTCTCGTTGTAC	CA_{18}	5	0.30	52.5	148 - 182	11	0.77	0.76	250
Oligr20	F TTGCTGCTTCTATCCCTTCTC R TGGTGTGCCTTGTCAATAGTC	$CT_4CA_{14}(GAA)CA_5$	5	0.25	52.5	271 - 319	11	0.74	0.76	250

PCR multiplex set refers to loci that co-amplify. T_A refers to annealing temperature, H_{obs} and H_{exp} refer to mean observed and gene diversity respectively across groups of skinks.

Ten tetranucleotide and five dinucleotide loci were isolated and used to amplify DNA from grand skinks (Table 1). Individuals were genotyped using DNA isolated from toeclips or tail-tips (*ca.* 2 mg tissue) by a salting-out procedure (Sunnucks & Hales, 1996) or Aquapure tissue isolation kit (Biorad). Two hundred and ninety grand skinks were genotyped at 15 loci on an ABI PRISM 377 sequencer using GENESCAN version 3.1 software (Perkin-Elmer). These loci were highly variable. The average number of alleles per locus was 15.73 (± 1.16 SE), and the average observed heterozygosity was 0.76 (± 0.03 SE). Tetranucleotide loci had significantly higher observed heterozygosity than dinucleotide loci ($P < 0.01$, two-sample permutation test with 9999 iterations).

I used the randomisation approaches implemented in FSTAT 2.9.3 (Goudet, 1995) to test for departures from Hardy-Weinberg and linkage equilibrium. Grand skinks live on rock outcrops in groups of *ca.* 20 related individuals and are isolated from other groups by 50-100 m of non-habitat matrix vegetation. This situation is likely to produce non-random associations between alleles. To account for Wahlund effects, Hardy-Weinberg tests were performed by randomising alleles among individuals on each rock outcrop. Using a global test, there was no significant overall departure from Hardy-Weinberg equilibrium ($P > 0.05$). However, tests per locus and per rock outcrop showed that on some rock outcrops the loci Oligr2 and Oligr15 had significant deficits of heterozygotes ($P < 0.01$; Oligr2 average $F_{IS} = 0.06$; Oligr 15 average $F_{IS} = 0.30$), and null homozygotes indicating the existence of null alleles. Loci were not significantly in linkage disequilibrium ($P > 0.05$).

New Zealand has two endemic skink genera, *Oligosoma* (~22 spp.), and *Cyclodina* (~7 spp.), which are members of the Eugongylus group of Indo-Pacific skinks (Hutchinson & Donnellan, 1993). Cross-species amplification of loci was tested on 16 of these 29 species and the related species, *Leiolopisma telfairi*, from Mauritius in the western Indian Ocean, and *Lampropholis delicata* from eastern Australia. Amplification fragments were considered homologous to *O. grande* microsatellites if they amplified fragments of similar size to those expected from *O. grande* (± 100 bp). I tested one individual of each species for each locus using the PCR profile optimised for *O. grande*. An average of 11.8 loci amplified in each New Zealand species tested (range 9-14; Table 2). Presumptive homologous loci also amplified in *L. telfairi* and

L. delicata suggesting that these markers could also be applied to other Eugongylus group skinks.

Although their use is now widespread, there have been few applications of microsatellite DNA in the conservation of New Zealand's biodiversity. The markers described here are highly variable in the grand skink and should be suitable for high-resolution genetic studies on other New Zealand skinks, including studies of dispersal, mating systems, and assigning provenance to smuggled animals. They are likely to find applications in ecological and conservation-related research on this diverse but imperilled fauna.

Table 2. Amplification of microsatellite DNA loci in New Zealand skinks (Genera: *Oligosoma* and *Cyclodina*). ● indicates amplification of a product of comparable size to that amplified from *O. grande*. ○ indicates no amplification of any products. One individual of each species was tested per locus. * denotes species with high conservation priority (Towns et al. 2001).

Species	Locus	Olgr1	Olgr2	Olgr3	Olgr4	Olgr6	Olgr7	Olgr8	Olgr10	Olgr11	Olgr13	Olgr14	Olgr15	Olgr17	Olgr19	Olgr20
Oligosoma																
*grande**		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
acrinasum		●	●	●	●	○	○	●	●	○	○	●	●	●	●	○
chloronoton		●	●	●	●	●	●	●	●	○	●	●	○	●	●	●
*homanolotum**		○	●	●	●	●	○	●	●	○	●	●	●	●	●	●
*infrapunctatum**		●	●	●	●	●	●	●	●	○	●	●	●	●	●	○
lineocellatum		●	●	●	●	●	●	●	●	○	●	●	●	●	●	●
*longipes**		●	●	●	○	●	●	●	●	○	●	●	○	●	●	●
moco		●	○	●	●	●	○	●	●	○	●	●	●	●	●	●
*otagense**		○	○	●	●	●	●	●	●	○	○	●	●	○	●	○
smithi		○	●	○	○	●	●	●	●	○	●	●	●	○	●	●
suteri		●	●	●	●	○	○	●	●	○	○	●	○	○	●	●
*waimatense**		●	●	●	●	●	●	●	●	○	●	●	●	●	●	○
zelandicum		●	○	●	○	●	○	●	●	○	●	●	●	○	●	●
Cyclodina																
aenea		●	○	●	●	●	●	●	●	○	●	●	○	●	●	●
*alani**		●	●	●	●	●	●	●	●	○	●	●	●	●	●	●
oliveri		●	●	●	●	●	○	●	●	○	●	●	●	●	●	●
*whitakeri**		●	●	●	●	●	●	●	●	○	●	●	○	●	●	●
Lampropholis delicata		○	○	○	○	○	○	●	○	○	●	○	○	○	○	○
Leiolopisma telfairi		○	○	○	●	○	○	●	●	○	○	○	●	○	●	○

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3

Can assignment tests measure dispersal?*

Abstract

Individual-based assignment tests are now standard tools in molecular ecology and have several applications, including the study of dispersal. The measurement of natal dispersal is vital to understanding the ecology of many species, yet the accuracy of assignment tests in situations where natal dispersal is common remains untested in the field. I studied a metapopulation of the grand skink, *Oligosoma grande*, a large lizard from southern New Zealand. Skink populations occur on isolated, regularly-spaced rock outcrops and are characterised by frequent inter-population dispersal. I examined the accuracy of assignment tests at four replicate sites by comparing long-term mark-and-recapture records of natal dispersal with the results of assignment tests based on microsatellite DNA data. Assignment tests correctly identified the natal population of most individuals (65-100%, depending on the method of assignment), even when inter-population dispersal was common (5-20% dispersers). They also provided similar estimates of the proportions of skinks dispersing to those estimated by the long-term mark and recapture data. Fully and partially Bayesian assignment methods were equally accurate, but their accuracy depended on the stringency applied, the degree of genetic differentiation between populations, and the number of loci used. In addition, when assignments required high confidence, the method of assignment (fully or partially Bayesian) had a large bearing on the number of individuals that could be assigned. Because assignment tests require significantly less fieldwork than traditional mark-and-recapture approaches (here < 3 months vs. > 7 years), they will provide useful dispersal data in many applied and theoretical situations.

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Introduction

Most organisms live in patchy environments. Ecologists seeking to understand how species persist in these environments usually estimate four key population parameters: rates of birth, death, emigration, and immigration (Hanski, 2001). Birth and death rates are typically measured by direct observation, often based on mark-and-recapture. Emigration and immigration (dispersal) can also be measured by direct observation, but estimation with genetic markers is another important and developing approach (e.g. Peacock & Smith, 1997).

Animal dispersal and gene flow have been measured with genetic markers for over sixty years (e.g. Dobzhansky & Wright, 1943). These parameters are often estimated indirectly from the spatial distribution of genetic variation among populations under the assumptions of Wright's island model (Wright, 1931; Neigel, 1997). This approach does not distinguish between contemporary and historical gene flow and dispersal, making it difficult to integrate such estimates with the instantaneous rates typically recorded in ecological studies (e.g. birth and death rates). Other limitations of using Wright's island model to measure dispersal are reviewed by Whitlock & McCauley (1999). In contrast, individual-based assignment tests, which assign individuals probabilistically to candidate populations by their multilocus genotype, are widely believed to hold the potential to estimate contemporary rates of gene flow and dispersal (Waser & Strobeck, 1998), and have rapidly become standard tools in molecular ecology.

Most tests of the ability of assignment tests to identify the source of individuals have concentrated on situations where recent dispersal is unlikely (e.g. Manel *et al.*, 2002). Typically in these situations, populations are distant and well differentiated genetically, and assignment has proven a useful tool for assigning provenance to trophies or smuggled animals, tracking the translocation history of endangered species, and as a general indicator of gene flow between populations (e.g. Paetkau *et al.*, 1995; Nielsen *et al.*, 1997; Manel *et al.*, 2002). Although these studies have shown that assignment can be highly accurate, they tell us little about the effectiveness of assignment in detecting ongoing dispersal in natural populations.

Dispersal is an important process for species inhabiting patchy environments because it influences both within- and between-patch population dynamics (Turchin,

1998). Consequently, measuring dispersal is a major focus of research in conservation biology, pest management, and evolutionary biology (e.g. Clobert *et al.*, 2001). Because dispersal is notoriously difficult and time consuming to measure directly (Koenig *et al.*, 1996), it has been suggested that in some situations assignment could replace field measures of dispersal such as mark-and-recapture (Waser & Strobeck, 1998; Cornuet *et al.*, 1999). However, in order to replace field measures, assignment methods first require verification. Thus far, attempts to verify the usefulness of assignment in cases where dispersal is common have been confined to simulation studies (e.g. Cornuet *et al.*, 1999), without comparison to empirical field data. Computer simulations in which the probability of correct assignment is measured in relation to dispersal rates have established that as dispersal rate increases, and genetic differentiation decreases, the ability to distinguish the source of an individual also falls. Further, they indicate that estimates of the level of genetic differentiation between populations (i.e. F_{ST}) are useful predictors of the performance of assignment methods that could be taken into account when designing research to measure dispersal (Cornuet *et al.*, 1999). However, the assumptions and results of computer models should be validated with empirical data (Brook *et al.*, 2000).

In this study I test the effectiveness of assignment methods for measuring natal dispersal in a situation where populations are well connected by dispersing animals. The species investigated, *Oligosoma grande* (the grand skink), is a rare lizard endemic to montane tussock grasslands in southern New Zealand. The skink lives in small groups of about 20 individuals that are restricted to free-standing rock outcrops, and separated from other groups by 50 - 150 metres of inhospitable vegetation. *Oligosoma grande* persists in this patchy environment by the formation of a metapopulation, with regular dispersal between rock outcrops (Whitaker, 1996). The species is well suited to investigating the accuracy of assignment tests because in addition to having clearly demarcated and easily censused populations, it has been the focus of a detailed long-term (>13 years) mark-and-recapture study (Whitaker 1996; M. Tocher, Department of Conservation pers. comm.).

Here I compare records of skink natal dispersal generated by mark-and-recapture with estimates from several commonly applied assignment tests based on microsatellite DNA data. Specifically, I ask: (1) how accurate is assignment in detecting the natal origin of individuals when compared to mark-recapture data, (2) how do the accuracies

of different assignment tests vary, and (3), how well does F_{ST} predict the accuracy of assignment?

Methods

Field methods

The study site was "Redbank", near Macraes Flat, southern New Zealand (45° 25'S, 170° 24'E; Figure 1). Part of the area is a reserve for the conservation of lizards, and consists of a mosaic of native montane tussock grasslands and agricultural pasture, punctuated by distinctive house-sized schist rock outcrops. Grand skinks are large lizards (to 111 mm snout-vent length) that inhabit these highly creviced outcrops, and rarely venture into the surrounding matrix vegetation (Whitaker, 1996).

I sampled four replicate sites, each of which consisted of a cluster of neighbouring rock outcrops (Table 1). Sites were chosen because they had large resident skink populations and were relatively isolated from other inhabited rocks. Two sites, P1 and T1, were studied during annual field seasons between 1995 and 2001 (M. Tocher pers. comm.). Each field season was divided into three repeat capture periods, which enabled near complete capture of resident skinks on each rock outcrop (Chapter 4). I also conducted intensive surveys at two additional sites, P2 and T2 during the 2000/2001 summer field season, and skinks were captured sporadically at these sites between 1995 and 2001 (M. Tocher pers. comm.). In most cases there were no inhabited rocks between the sampled rock outcrops, but at site P2 a small number of skinks (≤ 5) were present on rocks between the sampled rock outcrops.

Skinks were captured with nylon fishing line nooses and marked with a unique and permanent toe-code and released at their point of capture. During the 2000/2001 field season, tissue samples were collected for genetic analyses from all captured skinks. Samples consisted of toe-clips or tail-tips (< 2 mg tissue), and were placed immediately into liquid nitrogen. A total of 261 skinks were genotyped for 13 loci following the methods described in Chapter Two. This sample contained virtually all skinks inhabiting each rock outcrop based on mark-recapture estimates of population sizes (Chapter 4). I calculated the average number of alleles, the observed heterozygosity, and the average gene diversity per rock outcrop using FSTAT 2.9.3 (Goudet, 1995).

Identifying natal dispersers with mark-recapture data

I identified the natal rock of 131 genotyped skinks from their mark-recapture history. These skinks were first captured in autumn as newborns or in the following spring as young of the year. They could be distinguished because grand skinks have four easily

recognisable age/size classes based on their snout-vent length: ≤ 1 year old, ≤ 2 year old, ≤3 year old, and adults (Whitaker, 1996). I assumed that ≤ 1 year old skinks were captured on their natal rock. This assumption is based on the movement patterns of ≤ 1 year old skinks established from a more extensive mark-recapture dataset including additional sites, where only 3.3 percent of skinks first caught as newborns in autumn dispersed to another rock during their first year (n = 120; M. Tocher pers. comm.). I used this natal information to categorize skinks as either a natal disperser or natal stayer.

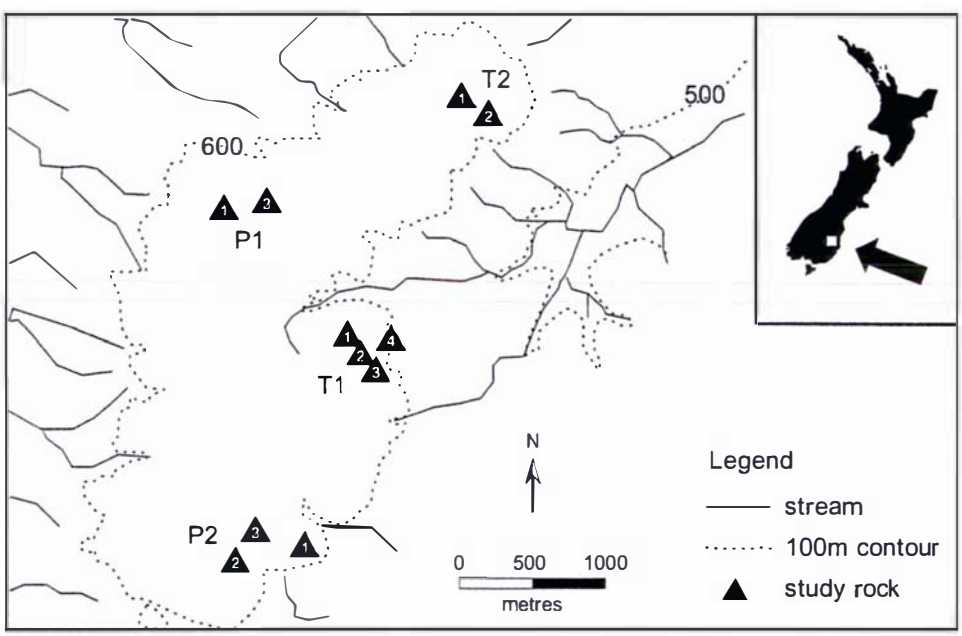

Figure 1. Macraes Flat, southern New Zealand, showing the location of the four replicate sites and their study rocks at "Redbank". Rocks within sites are separated by between 34 and 356 metres, and sites are separated by between 840 and 2460 metres.

Identifying natal dispersers with genotypic data

I used the genotypic data from all skinks to calculate the allele frequencies on each rock outcrop, and used two methods to assign individuals probabilistically to candidate rock outcrops based on their multilocus genotypes: the partially Bayesian method (Rannala & Mountain, 1997), and the fully Bayesian clustering method (Pritchard *et al.*, 2000).

The partially Bayesian method has been shown to be the most accurate of the 'frequentist' assignment approaches (Cornuet *et al.*, 1999), while empirical studies suggest the fully Bayesian method has equal or higher accuracy (e.g. Eldridge *et al.*, 2001). Both of these methods assume Hardy-Weinberg equilibrium and also linkage equilibrium between loci within each population. I performed separate assignment tests for each site, and also conducted assignment tests between all pairs of rock outcrops (both between and within sites). This permitted examination of the performance of assignment tests at both small and large spatial scales, where natal dispersal is common and rare respectively. The accuracy of assignment was calculated as the percentage of skinks with known natal rocks that were correctly assigned to their natal rock. I examined whether the same individual skinks were consistently assigned (using the most likely criteria) correctly or incorrectly to their natal rock by both assignment methods using the ϕ_2 correlation coefficient as a measure of association (Zar, 1996). ϕ_2 varies between 0 if there is no association between assignments by each method, and 1 or -1, if there is complete agreement or disagreement of assignments respectively. The significance of the association can be assessed by considering the significance of the contingency table with the chi-square statistic (Zar, 1996).

Assignment calculations

Partially Bayesian assignments were calculated with GENECLASS (Cornuet *et al.*, 1999) using the 'leave one out' option, which sequentially removes the individual under consideration when calculating the allele frequencies for each rock outcrop. Fully Bayesian assignments were calculated using STRUCTURE 2.0 (Pritchard *et al.*, 2000). STRUCTURE permits inclusion of a range of prior information as parameters in the model. I included an estimate of the probability that each individual was a migrant to the rock upon which it was captured (ν). Field data for skinks with known natal rocks indicated that between approximately 5-20% of skinks captured on a rock outcrop were born on another rock at the same site (M. Tocher pers. comm.; O. Berry unpubl. data). For STRUCTURE analyses within each site (small spatial scales) I accounted for uncertainty in these estimates by specifying two probabilities that each individual was a migrant ($\nu = 0.05$ and $\nu = 0.20$) in replicate runs of the analyses. No dispersal between sites was recorded in this study, but dispersal of up to 403 metres has been recorded previously (Whitaker, 1996), and it is likely that occasional longer dispersal events occur. In light of this, and because setting ν greater than zero is recommended by Pritchard *et al.* (2000), I set $\nu = 0.01$ for all between site (large spatial scale) runs of

STRUCTURE. For analyses involving pairs of populations I set the number of populations (K) = 2, and for assignments at site T1 and P2, which have four and three rocks respectively I set K = 4 or 3. In addition, I specified that the model use the rock outcrop where skinks were caught as prior information (USEPOPINFO = 1), and that the allele frequencies on different candidate rock outcrops were correlated (FREQSCORR = 1). The remainder of the input parameters were left at default values. When running STRUCTURE I used a burnin of 50,000 iterations of the Markov chain followed by a run for 50,000 iterations because it produced consistent results in replicate runs.

Stringency of assignments

Both the fully and partially Bayesian methods of assignment provide a level of confidence in the assignment of each individual. This permits *a priori* specification of the level of confidence, or stringency, required to accept an individual as a natal disperser or stayer. The chosen level of stringency will depend on the application of the data. For example, a very high level of confidence in an assignment may be required if the information is to be used in a court of law (e.g. $P = 0.001$; Primmer *et al.*, 2000), but a lower level of confidence may suffice in studies of wildlife dispersal (e.g. Pritchard *et al.*, 2000; Galbusera *et al.*, 2000). I calculated the accuracy of assignments under a range of stringencies for both methods of assignment as follows.

For the partially Bayesian analysis, skinks were assigned to candidate rocks at: 1) the most likely rock, 2) rocks with ≥ 80% confidence of exclusion ($P \leq 0.20$), and 3) rocks with ≥ 95% confidence of exclusion ($P \leq 0.05$). The exclusion method was introduced by Cornuet *et al.* (1999), and involves simulating a large number of multilocus genotypes from a rock's observed allele frequencies and comparing the probability that a focal skink's genotype originated on a candidate rock to a distribution of probabilities derived from the simulated genotypes. I performed these calculations with GENECLASS using 10,000 simulated individuals, and considered an assignment correct when only the known natal rock had a probability greater than or equal to 80% or 95% of being the source of a skink. This approach does not assume that the true candidate population has been sampled because it does not compare populations, but considers each separately. This may be advantageous in situations where sampling all of the candidate populations is not possible (Cornuet *et al.*, 1999).

For the fully Bayesian clustering analysis, skinks were assigned using STRUCTURE 2.0 to rocks at three levels of stringency: 1) the rock with the highest posterior probability of belonging, 2) rocks with a ≥ 95% posterior probability of belonging (T ≥ 0.95), and 3) rocks with a ≥ 99% posterior probability of belonging (T ≥ 0.99). In contrast to the partially Bayesian method, the fully Bayesian method assumes that the true candidate population included in the analysis, and the posterior probabilities that an individual originated from each of the candidate populations sum to one.

The relationship between genetic differentiation and the accuracy of assignment

I ran separate assignment tests for all pairs of rock outcrops, and calculated the percentage of skinks assigned correctly to their natal rock under each level of stringency. These data were plotted against F_{ST} values between pairs of rocks derived by the method of Weir & Cockerham (1984) using FSTAT 2.9.3 (Goudet, 1995). When conducting pairwise assignment tests some skinks being considered were known to have migrated from non-candidate rocks, thus automatically being incorrectly assigned to one of the candidate rocks. I removed these individuals when calculating the accuracy of assignment.

The effect of the number of loci on the accuracy of assignment

The effect of the number of loci used on the accuracy of assignments was tested. I confined these analyses to sites P1 and T1 because a large proportion of skinks had known natal rocks at these sites. In addition, these sites differ in their rates of natal dispersal, and have moderate and low levels of genetic differentiation respectively (P1 F_{ST} = 0.109; T1 F_{ST} = 0.041). I randomly sub-sampled without replacement five replicate datasets of 1 to 12 loci from the existing dataset and calculated the percentage of skinks correctly assigned to their natal rock for each sub-sample.

Results

Frequency of dispersal detected by mark-and-recapture

No between-site dispersal events were recorded by mark-and-recapture during this study, but dispersal was recorded between rock outcrops at all of the sites. The majority of skinks remained on their natal rock, but the percentage of skinks that were natal dispersers ranged between 5 and 18.9 percent at different sites. The mean dispersal distance recorded for dispersing skinks with known natal rocks was 118.4 m (± 20.6 SE, n = 13 dispersers). The longest dispersal distance recorded was 235 m at site P1.

Genotypic data

I genotyped between 15 and 38 skinks per rock outcrop (Table 1). The populations on each outcrop were highly polymorphic for the microsatellite loci examined, with the average number of alleles per locus on each rock outcrop ranging between 6.2 and 9.0. The average gene diversity ranged between 0.73 and 0.80 (Table 1). Global and by-rock tests showed that the loci were in Hardy-Weinberg equilibrium and in linkage equilibrium.

Table 1. Summary statistics for 13 microsatellite loci from 11 grand skink populations at Redbank, southern New Zealand.

Site	Rock	*Site* F_{ST}	*n*	*N natal*	*A*	H_{obs}	H_{exp}
P1	1	0.109 ± 0.021	25.0 ± 0.0	14	7.8 ± 0.5	0.717 ± 0.159	0.774 ± 0.038
	2		37.4 ± 0.3	26	9.0 ± 0.8	0.803 ± 0.108	0.776 ± 0.020
T1	1	0.041 ± 0.006	22.7 ± 0.4	14	8.2 ± 0.7	0.800 ± 0.180	0.793 ± 0.045
	2		21.1 ± 0.3	15	8.1 ± 0.8	0.816 ± 0.082	0.794 ± 0.022
	3		22.0 ± 0.0	15	8.4 ± 0.6	0.860 ± 0.178	0.800 ± 0.040
	4		15.9 ± 0.1	9	8.2 ± 0.7	0.820 ± 0.193	0.800 ± 0.042
P2	1	0.073 ± 0.011	28.6 ± 0.2	13	7.9 ± 0.5	0.834 ± 0.122	0.780 ± 0.026
	2		26.4 ± 0.1	3	6.8 ± 0.4	0.766 ± 0.214	0.728 ± 0.051
	3		18.3 ± 0.2	5	6.2 ± 0.0	0.791 ± 0.188	0.741 ± 0.045
T2	1	0.053 ± 0.006	22.0 ± 0.7	5	7.5 ± 0.5	0.811 ± 0.176	0.768 ± 0.045
	2		15.0 ± 0.0	12	6.8 ± 0.4	0.744 ± 0.207	0.760 ± 0.050

Site F_{ST} = site-wide F_{ST}, *n* = average number of skinks genotyped at each locus, *N natal* = number of skinks with known natal rocks, *A* = average number of alleles at each locus, H_{obs} = average observed heterozygosity at each locus, H_{exp} = average gene diversity at each locus (Nei 1987). All estimates are ± standard error.

The accuracy of assignment tests to detect natal dispersal and the effect of high stringency

Both the partially and fully Bayesian assignment tests without high stringency correctly identified most natal dispersers and stayers at all of the sites (Figures 2a & 2b). The least accurate result was the fully Bayesian method at site T2, which assigned 64.70 percent of skinks correctly to their natal rock. Both methods assigned more than 95 percent of skinks correctly to their natal rock at sites P1 and P2 and also for the between-site analysis.

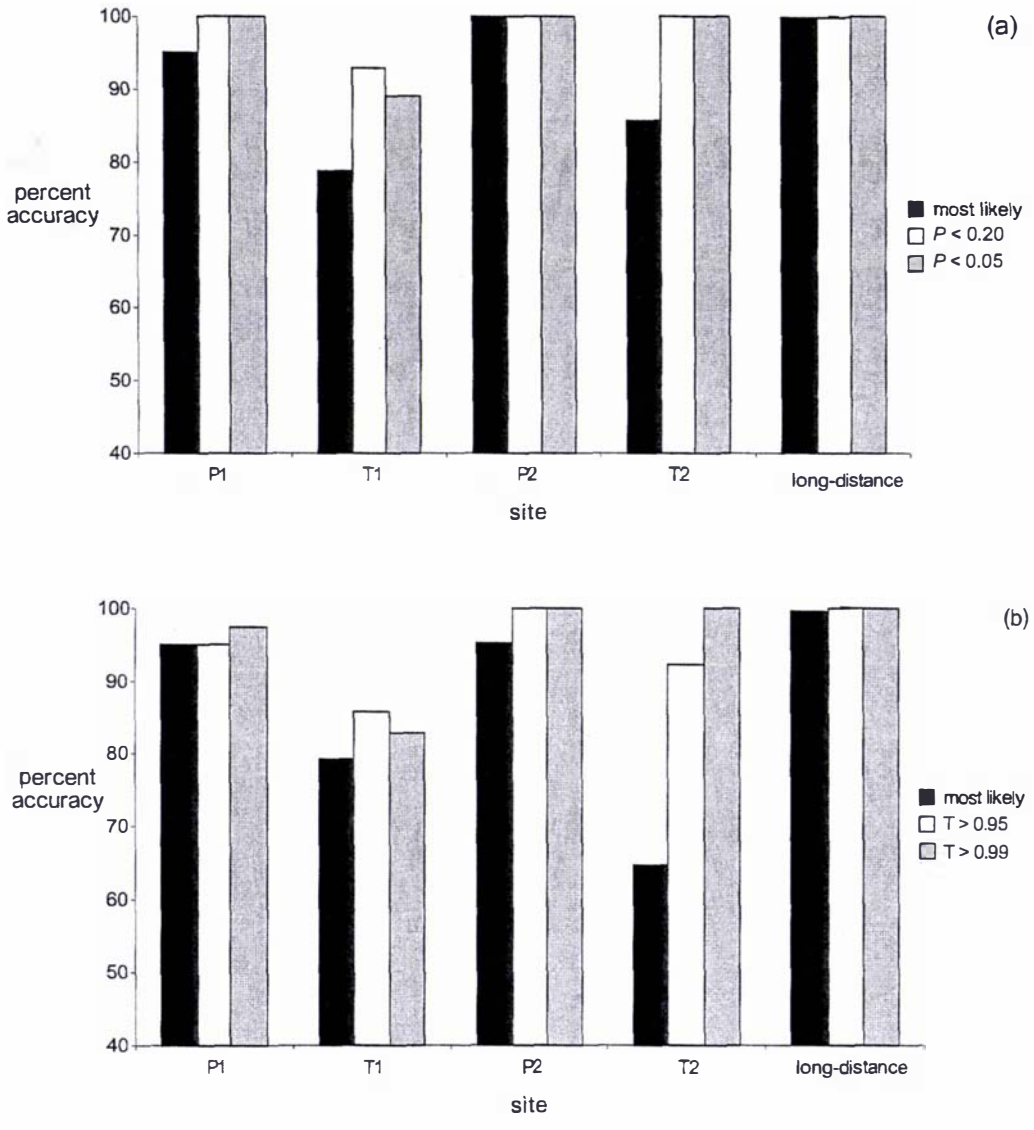

Figure 2. Percent of individual skinks correctly assigned to their natal rock at each site, and correctly assigned to their natal rock in pairwise tests between study rocks from different sites (long-distance, average ± SE). a) partially Bayesian method, b) fully Bayesian method.

Assignments that met the stringency criteria were more accurate than assignments without high stringency at all sites (Figures 2a & 2b). The partially Bayesian method achieved 100 percent accuracy for both the 80% ($P \leq 0.20$) and 95% ($P \leq 0.05$) exclusion at all sites except T1, where they achieved 70 and 89 percent accuracy respectively. Both stringencies of the fully Bayesian method achieved over 92% accuracy at all sites and for the between-site analysis, except at site T1 where they achieved 85 and 82 percent accuracy respectively.

Although assignments with higher stringency were more accurate, fewer skinks could be assigned because more had undefined origins (Figure 3). This was most pronounced for the partially Bayesian exclusion method. At all sites and also for the between-site analysis fewer than 40 percent of skinks were assigned to one rock outcrop with $P \leq 0.2$ and fewer than 30 percent were assigned with $P \leq 0.05$. For the fully Bayesian method, the effect of a stringency of $T \geq 0.99$ and 0.95 was less marked. At all of the sites except T1 more than 90 percent of skinks were assigned with a posterior probability ≥ 0.95, and at all of the sites more than 78 percent could be assigned with more than 0.99 posterior probability. At the larger, between-site scale, on average more than 99 percent of skinks were assigned with a posterior probability ≥ 0.99.

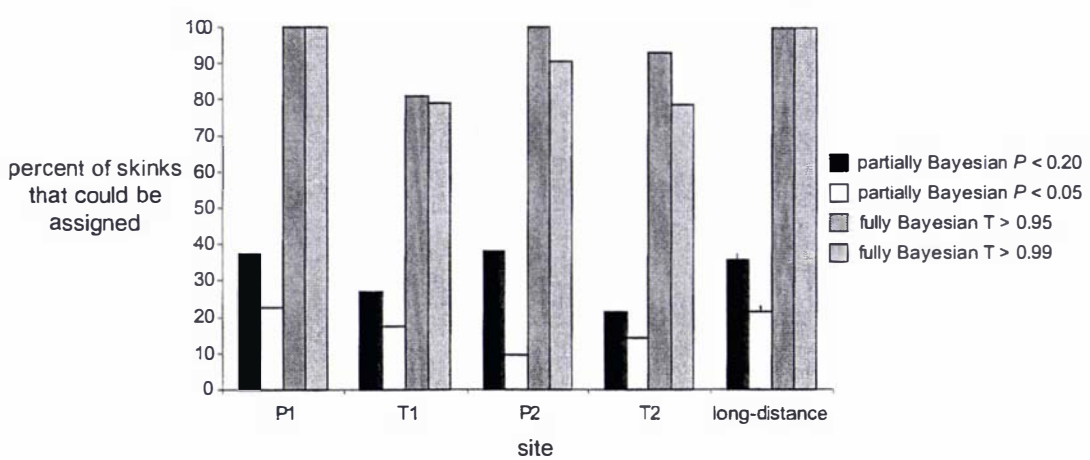

Figure 3. Percent of skinks that were assigned to a rock outcrop and met stringency criteria at each site and in pairwise tests between study rocks from different sites (long-distance, average ± SE).

Individual skinks at sites P1 and T1 were usually assigned in the same manner (correctly or incorrectly to their natal rock) by both assignment methods using the most likely criteria (site P1 $\phi 2$ correlation = 1.0, χ^2 (with Yates correction) = 21.72, d.f. = 1, $P < 0.001$, n = 40; site T1 $\phi 2$ correlation = 0.46, χ^2 (with Yates correction) = 8.49, d.f. = 1, $P < 0.004$, n = 53). Sample sizes were too small for valid tests to be performed for the other sites (P2 and T2). Under the most likely stringency, neither method of assignment was consistently more accurate at all four sites (compare Figures 2a & 2b), and there was no significant difference in the percentage assigned correctly by each method in the between-site analyses (paired t-test, $t = 1.54$, d.f. = 54, $P = 0.13$, n = 55).

The effect of the number of loci on the accuracy of assignment

The accuracy of partially Bayesian assignments usually improved when more loci were included, but the rate of improvement differed for sites T1 and P1 (Figures 4a & 4b). The improvement was gradual at site T1, which has a low level of genetic subdivision ($F_{ST} = 0.045$), and improved from *ca.* 50 percent with one locus to 78.8 percent with 13 loci. In contrast, assignment accuracy improved rapidly at the more genetically subdivided site P1, starting between 70 and 90 percent and reaching an asymptote at 95 percent with close to four loci. In addition, while the combination of loci used caused the accuracy to vary by often more than 10 percent at site T1, the level of accuracy varied little between loci combinations at site P1. In contrast to the partially Bayesian analysis, the accuracy of fully Bayesian analysis showed no improvement when more loci were included (Figures 4c & 4d), but using a more conservative prior probability of being a natal disperser ($\nu = 0.05$) usually produced slightly greater accuracy at both sites for all numbers of loci and loci combinations.

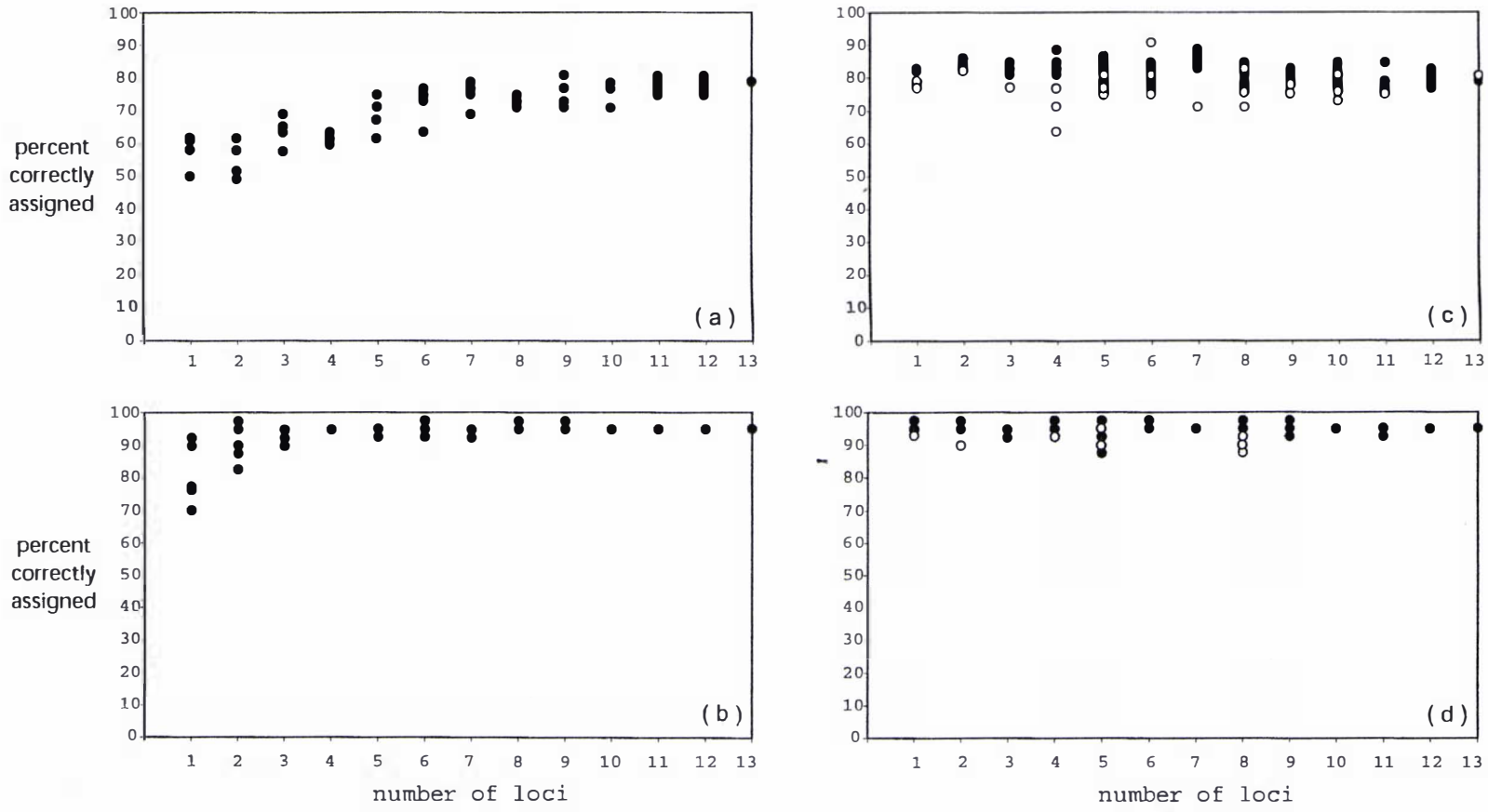

Figure 4. The relationship between the number of loci used for assignment tests and the percentage of assignments that were correct at: a) site T1, partially Bayesian method; b) site P1, partially Bayesian method; c) site T1, fully Bayesian method; d) site P1, fully Bayesian method. For the fully Bayesian analyses open diamonds represent tests where a prior migration probability (v) of 0.20 was specified and filled diamonds represent a prior migration probability of 0.05.

Estimating the number of dispersers from mis-assignments

If skinks assigned to rocks other than those upon which they were captured were considered to be dispersers (see Cornuet *et al.*, 1999, page 1999), both assignment methods estimated numbers of dispersers similar to those estimated by the mark-and-recapture method within each site (Figure 5). The greatest exception was at site T1, where the partially Bayesian and to a lesser extent the fully Bayesian method overestimated the proportion of individuals that were dispersers. The prior estimate of dispersal probability (ν = 0.05 or 0.20) generally had little effect on the estimate of the number of dispersers for the fully Bayesian method. While there were no inter-site dispersers recorded in the mark-recapture data, a small number of dispersers were identified by both the partially and fully Bayesian methods, which both on average estimated 0.13 (± 0.13 SE) percent of individuals dispersing between pairs of sites.

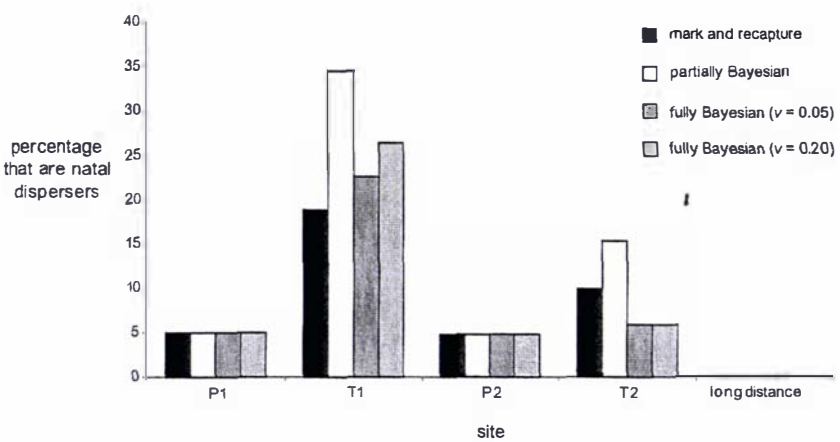

Figure 5. The percentage of skinks that were identified as natal dispersers by mark-and-recapture or that were assigned to a rock outcrop other than where they were captured at each site and between all pairs of rock outcrops between sites (long-distance, average ± SE). For the fully Bayesian analyses the results for two prior migration probabilities (ν) are shown.

The relationship between genetic differentiation and the accuracy of assignment

The percentage of skinks assigned correctly increased as populations were more genetically differentiated under both the partially and fully Bayesian assignment approaches (Figures 6a & 6b). Both methods with the most likely stringency reached close to their maximum accuracy of 100 percent at an F_{ST} between 0.06 and 0.08. Applying higher stringencies to the partially Bayesian analysis greatly reduced the proportion of skinks that could be assigned with sufficient confidence to any candidate rock outcrop, thus reducing the accuracy of this method, although skinks were not strictly assigned incorrectly (*cf.* Figure 3). Applying higher stringencies to the fully Bayesian method reduced the accuracy of assignment relatively little because most skinks were assigned to one of the candidate rocks rather than having undefined origins, and above F_{ST} of 0.07 only 4 of 44 comparisons were less than 100% accurate with $T \geq 0.99$, and all of these were more than 90% accurate.

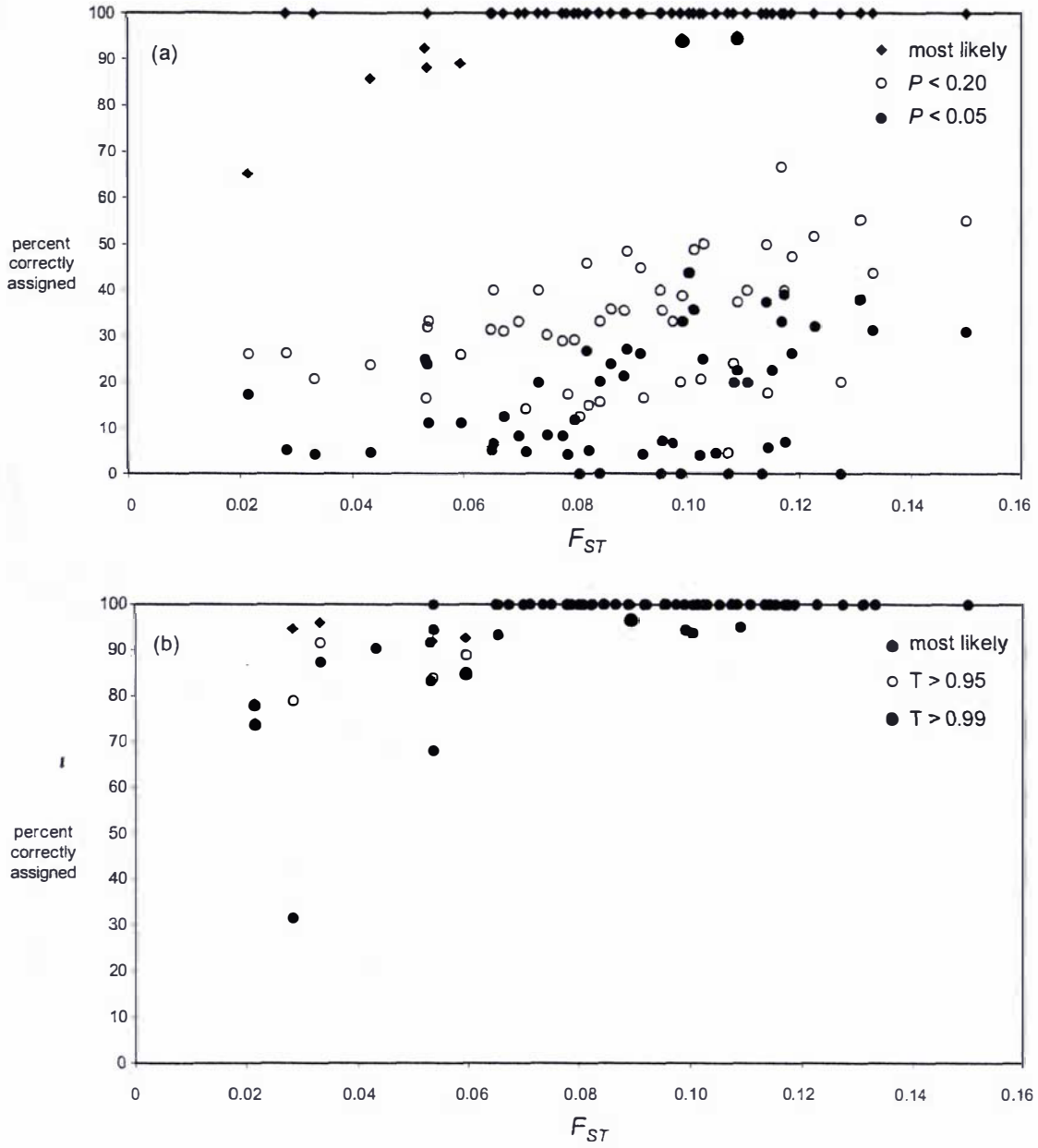

Figure 6. The relationship between genetic differentiation and the accuracy of assignments in pairwise tests between all rock outcrops. The effect of different stringencies on the percentage of correct assignments is also shown. a) partially Bayesian method, b) fully Bayesian method. In b), intra-site tests (predominantly $F_{ST} < 0.1$) were run with two prior migration probabilities. For simplicity only the results from the prior migration probability of 0.20 are shown. Assignments were usually more accurate for the $v = 0.05$.

Discussion

Genetic studies are often viewed as a quick means to measure dispersal (Steinberg & Jordan, 1998). For example, the field data necessary for the molecular component of this study were obtained in less than three months, whereas the mark-and recapture data were generated over more than seven years. If these methods produce similar results, then assignment tests would represent a major saving of effort and expense and reduce the invasiveness of field studies. The combined microsatellite DNA data and long-term mark-and-recapture records presented here provide strong evidence that assignment tests can provide dispersal data of high accuracy. They show that in most cases, assignment tests were reliable indicators of the origin of individuals, even when populations regularly exchanged migrants, but also that accuracy depended on the stringency applied, the level of genetic differentiation between populations, and the number of loci used.

Using assignment tests for qualitative and quantitative measures of dispersal

Assignment tests provided a qualitative idea of the spatial scale of skink dispersal consistent with expectations from mark-and-recapture studies. Very few skinks (average 0.13%) were assigned to a rock outside their site (distances 840-2460 metres), but more were assigned to a rock outcrop within their site (distances 34-356 metres), indicating that dispersal of distances greater than 800 metres is rare. This matches the pattern found in the mark-and-recapture data, and had little been known about rates of dispersal in grand skinks, this information would be valuable in defining the area necessary to establish a reserve for these endangered lizards (Whitaker & Houston, 2002). However, the consequences of dispersal for the dynamics of populations can best be understood with quantitative estimates of dispersal (Turchin, 1998). Therefore, one of the most important results was that assignment tests without high stringency provided estimates of the number of natal dispersers that were comparable to those obtained by mark-and-recapture. Like previous studies on rock wallabies and ibex (Eldridge *et al.*, 2001; Maudet *et al.*, 2002), assignment was accurate at the large spatial scale where natal dispersal must be very rare or absent, but importantly assignment was also accurate at each site where between 4.8 and 18.9 percent of skinks disperse from their natal rocks. In addition, at the level of individual skinks, the accuracy of assignment tests was high, with most individuals being assigned correctly to their natal rock. Although similarly high accuracy has been demonstrated previously, it has

usually been between more differentiated populations that exchange few migrants (Eldridge *et al.*, 2001; Manel *et al.*, 2002).

The effect of population differentiation on assignment accuracy

The data demonstrate a positive relationship between F_{ST} and the ability to correctly assign individuals to their natal population, similar to predictions made from simulations using both infinite allele and stepwise mutation models (Cornuet *et al.*, 1999). Hence assignment tests appear robust to assumptions about the model of mutation for the markers, and have general application. I also showed that assignments were usually 100% accurate for low to moderate levels of genetic differentiation (F_{ST} ~ 0.07), and still moderately accurate (~ 78%) for population pairs with fairly low levels of genetic subdivision (e.g. site T1, F_{ST} = 0.04) that correspond to reasonably high levels of natal dispersal (18.9%). These results indicate that assignment methods will have wide applicability in population studies.

Assignment results were more accurate than those from previous empirical studies. In the study by Maudet *et al.* (2002) for reintroduced ibex populations, where F_{ST} between populations were usually greater than those under study here (F_{ST} = 0.167), assignments were 74.4% accurate for the fully Bayesian method with T ≥ 0.99. Similarly, across a range of datasets where F_{ST} values were usually greater than those reported here (average = 0.270), the mean percentage of individuals correctly assigned by the fully Bayesian method at T ≥ 0.99 was also lower (*ca.* 75%; Manel *et al.*, 2002). Notably, the heterozygosities reported for loci in those studies were lower than the values I recorded (averaging 0.40 and 0.47 respectively, versus 0.77), supporting the notion that loci with higher levels of variation (measured by number of alleles or gene diversity) are better at discriminating the sources of individuals (Estoup *et al.*, 1998; Bjørnstad & Røed, 2002). Thus, where possible, highly variable loci should be used in assignment analyses.

The number of loci required for assignment

Simulation studies have shown that additional loci improve the ability to correctly assign individuals for a given level of differentiation between populations (Cornuet *et al.*, 1999). Conceivably, assignment could accurately measure higher rates of dispersal if sufficient loci were used. Yet the results show that the rate of improvement with the addition of loci is gradual when genetic differentiation is low, and suggest that many

loci would be necessary for species where dispersal is high, possibly incurring high financial costs and so diminishing any potential advantage over field based measures of dispersal. Currently, most microsatellite DNA-based molecular surveys use fewer than the 13 loci used here; the average number used to study animal population genetic structure was 8.00 (± 0.48 SE, n = 35) in *Molecular Ecology* publications in 2001. Because there are many organisms for which molecular markers have not been developed, the additional cost and time required for marker development may limit the usefulness of genetic measures of dispersal. However, as more efficient protocols for microsatellite development are used and co-amplification of multiple loci becomes more common, the cost is likely to decrease (Henegariu *et al.*, 1997; Zane *et al.*, 2002).

Different methods of assignment and the effect of high stringency

Testament to the usefulness of assignment tests is the rapid proliferation of methodological variants that have been developed (e.g. Bowcock *et al.*, 1994; Rannala & Mountain, 1997; Pritchard *et al.*, 2000). I found that the fully Bayesian approach of Pritchard *et al.* (2000) and the partially Bayesian approach of Rannala & Mountain (1997) usually produced equally accurate assignments when the most likely criterion was applied. Both were also highly accurate at higher stringencies, but the methods differed greatly in the proportion of individuals that they could assign with these higher stringencies. In part this is explained by an important difference in the assumptions behind the derivation of confidence in these assignment methods; the fully Bayesian method assumes that the true candidate population has been sampled, whereas the partially Bayesian method does not. Because I had near complete knowledge of the provenance of all individuals, I could be confident that all the appropriate candidate rock outcrops were sampled, and I removed seven skinks from the analysis that were known to have dispersed from outside the study rocks. This fulfils a key assumption of the fully Bayesian method. When the skinks that originated from outside the study rocks were not excluded from the analysis, the partially Bayesian correctly excluded them all from the rock on which they were captured at $P \leq 0.05$, while the fully Bayesian method incorrectly assigned six of these skinks to the rock on which they were captured with high probability ($T \geq 0.99$). Comparison of these results is problematic because the assumptions of the two assignment methods differ. However, when the known outside-site skinks were included, the partially Bayesian method was a much more conservative approach to assignment with high confidence because only a small proportion of individuals could be assigned. Hence when assignments require high

confidence, the method of assignment can have a large bearing on the number of individuals that can be assigned. Further, the likelihood that all appropriate populations have been sampled should be an important consideration when deciding on the method of assignment to be used.

High stringency analyses are important in situations where the cost of an incorrect assignment is high, such as in forensic cases where legal action is undertaken (Primmer *et al.*, 2000). Although the results show that assignments with high stringency were more accurate than assignments without the stringency criteria, this advantage can be subtle, and must be traded against the sometimes large reduction in the number of individuals that can be assigned. In the case of the partially Bayesian methods, fewer than half of the skinks could be assigned to a natal rock. Manel *et al.* (2002) reported similarly high numbers of individuals of undefined origin, and simulations also show many un-assigned individuals (Cornuet *et al.*, 1999).

Comparing assignment data with mark-and-recapture data

Although assignment tests provided quantitatively similar estimates of dispersal rates to those provided by the field data, examination of the assignment of individuals reveals that sometimes up to one third of assignments were incorrect at sites with less genetic subdivision (*cf.* Figure 2a & 2b). Based on the field data, both natal dispersers and natal stayers were sometimes incorrectly assigned. These errors cancelled each other when the total number of 'dispersers' was tallied. In addition, quantifying the error rate is complicated by the possibility that errors exist in the mark-recapture data. My preliminary data showed that a small proportion (*ca.* 3%) of field-based natal classifications would be incorrect because some newborn skinks disperse in their first year. Furthermore, natural toe loss occurs occasionally in grand skinks and some individuals may be misidentified. Indeed, some individuals not identified as dispersers by the field data were consistently labelled dispersers by all assignment methods, and for many combinations of loci, indicating that their genotype was atypical for the rocks upon which they were resident. These data strongly suggest that some dispersal events were not detected by mark-recapture. Although I believe there were very few errors in the mark-and-recapture data, they inevitably do occur, and the assignment data provides a useful check of dispersal records. For example, one skink identified by the mark-and-recapture data at site P1 as a disperser was never identified as a disperser by assignment tests. As this site had a moderately high level of genetic subdivision (F_{ST} = 0.11), and

assignments were highly accurate, this leads us to suspect that an error was made in the field data. In addition, the offspring and descendents of dispersers will sometimes be incorrectly assigned because they will have genotypes typical of more than one population. This should inflate the number of mis-assigned skinks over the true dispersal rate, particularly when dispersal rates are high and matings with migrants are common (*cf.* Figure 5). If populations can be well sampled, one approach to identifying such individuals would be to use parentage or kinship analysis (Marshall *et al.*, 1998; Goodnight & Queller, 1999), in combination with assignment tests. This approach is investigated in Chapter Five (and see Telfer *et al.*, 2003).

Conclusions

Estimating rates of dispersal is critical to understanding the dynamics of patchy populations, yet dispersal is difficult and time consuming to measure (Koenig *et al.*, 1996). I have shown that in a short time period, assignment tests can identify the natal population of most individuals, and provide estimates of the proportion of migrants that are similar to results from a long-term mark-and-recapture study. Because assignment tests require significantly less fieldwork than traditional mark-and-recapture approaches to measuring dispersal (here < 3 months vs. > 7 years), they will provide useful qualitative and sometimes quantitative estimates of dispersal in many applied and theoretical situations. However, several caveats apply: (1) assignment tests are more accurate when populations are more genetically differentiated, although they can still be highly accurate at low to moderate levels of genetic differentiation when dispersal is common; (2) assignment accuracy can be improved by increasing the number of loci used, but if populations have a low level of genetic differentiation the number of loci required may be prohibitive; and (3), assignment accuracy can also be improved by applying high stringency, but the method used (fully or partially Bayesian) can have a large bearing on the number of individuals that can be assigned.

Acknowledgements

I thank Mandy Tocher for generously sharing her mark-recapture data with me. I also thank Jeroen Spitzen, Annemarieke van der Sluijs and Amanda Smale for assistance with skink catching, and Graeme Loh and Dave Houston for skink-related discussions. The manuscript was improved by comments from Hilary Millar, Nicola Mitchell, Nancy Fitsimmons, Christian Schlötterer, and two anonymous reviewers.

4

Effect of vegetation matrix on animal dispersal: genetic evidence from a study of endangered skinks*

Abstract

Maintaining connectivity in fragmented landscapes is a key principle of biological conservation. Although corridors are a widely accepted approach to connecting populations, their merits are still debated, and they may be impractical in many situations. A focus on management of the vegetation matrix between populations has been advocated as an alternative way to deal with habitat fragmentation and has theoretical support. I combined microsatellite DNA and demographic data to provide an empirical account of how two forms of agricultural land use affect the connectivity of insular populations of an endangered skink from southern New Zealand. The grand skink (*Oligosoma grande*), lives in small populations (*ca.* 20 individuals) on rock outcrops separated from one another by 50-150 m of inhospitable matrix vegetation (either native tussock grassland or exotic pasture). Skinks typically dispersed short distances, and the nature of the vegetation matrix both quantitatively and qualitatively affected dispersal dynamics. Skink populations in pasture were significantly more genetically structured and had less genetic variation than similar populations in tussock, implying less dispersal between populations in pasture than tussock. Furthermore, although female-biased dispersal was a feature of populations in tussock, no sex bias was evident in pasture. In addition, Bayesian individual-based genetic assignment tests that incorporated prior mark-recapture information revealed that some populations produced many emigrants but received few immigrants, whilst others were relatively insular. Patterns of dispersal and response to matrix vegetation were complex, and the causes of these patterns deserve attention in future studies of habitat fragmentation. Managing the vegetation matrix may be a practical way to connect animal populations in some situations.

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"The matrix matters."

Ricketts, 2001

Introduction

The maintenance of connectivity in fragmented landscapes is a long-standing principle of conservation biology and stems from the prediction that small, isolated populations risk stochastic extinction (Lande, 1988). Because many landscapes are now fragmented, determining how connectivity can best be promoted in these environments is the subject of concerted research (Beier & Noss, 1998; Tewksbury *et al.*, 2002). Much attention has been directed toward corridors, which are strips of high-quality habitat that permit animal dispersal between otherwise disconnected patches, but ecologists are increasingly focusing on the nature of the matrix outside habitat patches or corridors and investigating how the matrix influences connectivity in fragmented landscapes (Ricketts, 2001; Perfecto & Vandermeer, 2002).

Recent computer models that incorporate different types of matrix show that the nature of the matrix can profoundly affect the dynamics of fragmented populations. For example, Fahrig (2001) showed that increasing the quality of the matrix reduces the amount of patch habitat required for a species to persist. Other models demonstrate that by decreasing the resistance of the matrix and thus increasing interpatch dispersal, the overall likelihood of persistence of a metapopulation increases (Vandermeer & Carvajal, 2001). These models indicate that managing the matrix is theoretically a useful way to maintain connectivity in a fragmented landscape, but require empirical support.

In practice, the influence of the matrix on animal dispersal is likely to be species specific and depend in part on the dispersal ability of the target species (Ricketts, 2001). A key to understanding how a target species responds to the matrix is to measure dispersal in different landscapes. However, for most organisms long-term patterns of dispersal are difficult to measure by conventional means such as mark and recapture, and consequently, most studies compare only short-term patterns of dispersal (e.g. Roland *et al.*, 2000; Ricketts, 2001; Jonsen *et al.*, 2001). Yet short-term studies may not detect subtle differences in dispersal rates and may miss important dispersal events if the species are cryptic or if the events are rare (Koenig *et al.*, 1996).

Genetic studies based on patterns of genetic subdivision among populations are sometimes viewed as a rapid way to measure dispersal that can provide a greater temporal perspective on patterns of connectivity between populations (Steinberg & Jordan, 1998). Typically, investigators compare estimators of genetic structure, such as F_{ST}, θ, or R_{ST}, and estimators of genetic diversity, such as heterozygosity between populations in different landscapes, with the expectation that the more fragmented landscape should be more genetically structured and less genetically variable (e.g. Knutsen *et al.*, 2000; Mech & Hallett, 2001). Although this approach can show evidence of qualitative differences in dispersal between fragmented and unfragmented landscapes, it has at least three limitations. First, all the estimators can be slow to respond to changes in dispersal rates (Steinberg & Jordan, 1998; Whitlock & McCauley, 1999), making them least useful when perturbations to the landscape are recent (e.g. land clearing). These properties and their relevance to studies of landscape connectivity have been discussed at length in the literature (Sarre, 1995; Steinberg & Jordan, 1998; Whitlock & McCauley, 1999; Pannell & Charlesworth, 2000). Second, in providing a measure of dispersal that is applied to all individuals under study, these estimators may not recover the maximum information about dispersal by individuals that is available from highly variable multilocus genotypes (e.g. Paetkau *et al.*, 1995; Stow *et al.*, 2001). Finally, these approaches can generally only provide a qualitative estimate of dispersal because the assumptions required to interpret them quantitatively rarely hold (Whitlock & McCauley 1999). This makes it difficult to integrate the data with the quantitative and instantaneous demographic data such as birth and death rates that are usually obtained in ecological studies so they can be included in models of population dynamics.

Recent analytical developments in population genetics, in particular genetic assignment tests, can provide improved resolution of patterns of dispersal because they focus on the individual rather than the population (Waser & Strobeck, 1998). Applications include testing for sex-biased dispersal, identifying hybrid individuals, and identifying dispersing individuals (e.g. Favre *et al.*, 1997; Galbusera *et al.*, 2000; Beaumont *et al.*, 2001). A further advantage of the assignment approach is the ability to make use of existing demographic data to inform the genetic analysis (Pritchard *et al.*, 2000; Gaggiotti *et al.*, 2002), which together can provide highly accurate quantitative dispersal information (Chapter 3).

I investigated how two structurally different types of vegetation matrix influenced connectivity between populations of the endangered skink *Oligosoma grande* (the grand skink) in southern New Zealand. This large lizard is well suited to studying the effects of matrix on dispersal because it lives in easily censused groups of *ca.* 20 individuals on discrete, regularly spaced rock outcrops that are separated from other groups by 50–150 m of vegetation. Two types of vegetation separate rock outcrops: native tussock grassland (*Chionochloa spp.*), which dominated southern New Zealand following Polynesian settlement but is now greatly reduced in extent (McGlone *et al.*, 1995), and exotic pasture. Livestock graze both tussock grassland and pasture, but tussock grassland is denser and structurally more complex than pasture. Results of a previous study suggest that skink populations in pasture are less abundant, patchier, and more extinction prone than populations in tussock, possibly because pasture inhibits dispersal and recolonisation of rock outcrops (Whitaker, 1996). However, this remains untested because long-term patterns of dispersal have not been studied in *O. grande*.

I combined demographic data and data from microsatellite DNA markers to investigate connectivity of grand skink populations in different matrix vegetation. First, I characterized levels of genetic subdivision and genetic diversity in replicate tussock and pasture landscapes to qualitatively examine patterns of connectivity. Then I combined individual-based assignment analyses and mark-recapture data to reveal that patterns of connectivity may be more complex than is obvious from the qualitative genetic analysis alone.

Methods

Field methods

The study area was located at Redbank, near Macraes Flat in southern New Zealand (45° 25'S, 170° 24'E). Part of this area is a reserve for lizard conservation and consists of a mosaic of tussock grassland and agricultural pasture regularly punctuated by house-sized schist rock outcrops (Figure 1). I used nooses of nylon fishing line (see Patterson, 1992) to capture skinks from two pasture-dominated (P1 & P2) and two tussock-dominated sites (T1 & T2). Each site consisted of between two and four rock outcrops inhabited by skinks (e.g., P1.1, P1.2...T1.1, T1.2) and was chosen because it had large skink populations and the group of rocks was relatively isolated from neighbouring rocks. Sites were separated by distances > 800 m, which is larger than the maximum dispersal distance recorded for grand skinks (678 m; Houghton, 2000), so I considered the sites to be independent of one another. Skinks were captured between November 2000 and April 2001 and were marked with a unique and permanent toe code and released. I captured nearly all resident skinks on each outcrop by repeatedly visiting rock outcrops over the skink's entire active period (November – April). This was verified by estimation of the population sizes by mark-recapture analyses.

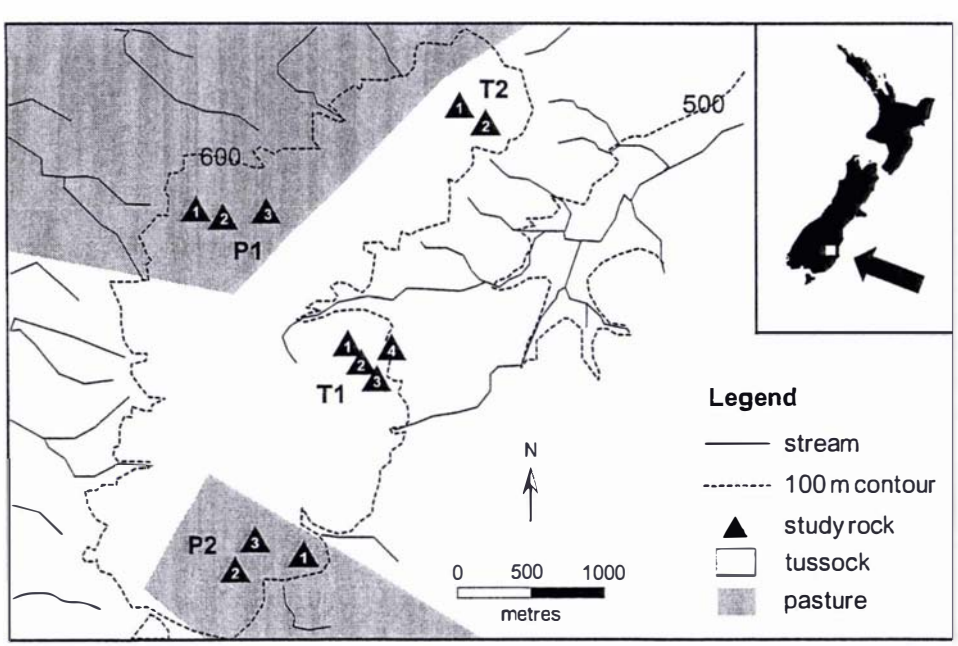

Figure 1. Study sites at "Redbank", near Macraes Flat, southern New Zealand showing the distribution of study rocks and tussock and pasture matrix types. In 1982 the entire study area was covered by tussock grassland; however, sites P1 and P2 were converted to pasture in 1983 and 1988 respectively.

Estimation of population sizes

I used a mark and resight method to estimate the number of skinks resident on five rock outcrops. Each rock was visited three to six times over a period of 7 to 15 days in April (Autumn). Skinks were captured on each visit and marked with a highly visible and unique number with a non-toxic marker. I also counted numbered and unnumbered skinks, noted their size class and mapped their location. Newly marked individuals were released after the completion of each survey. The combination of unique numbers, four easily distinguished size classes, fidelity to crevice retreats, and small population sizes minimized the chances of double counting individuals. Furthermore, because the survey period was brief and births are uncommon at this time, dispersal, deaths, and recruitment were highly unlikely. I analysed the data as a closed system by the Schumacher-Eschmeyer modification of the Petersen method (Krebs, 1999).

DNA and data analysis

Tissue samples from all captured skinks were collected for genetic analysis. Samples consisted of toe clips or tail tips (< 2 mg tissue) and were placed immediately into liquid nitrogen. The DNA was extracted by a salting-out procedure (Sunnucks & Hales, 1996). A total of 261 skinks were genotyped for 15 microsatellite DNA loci following methods described in Chapter Two.

I tested the data for departures from Hardy-Weinberg and linkage equilibrium with the randomisation approaches implemented in FSTAT 2.9.3 (Goudet 1995), and I calculated observed heterozygosity and gene diversity on each rock outcrop according to Nei (1987). As a measure of genetic subdivision, I calculated the correlation of gene frequencies (F_{ST}) among all rock outcrops and between all pairs of rock outcrops by the method of Weir and Cockerham (1984) with FSTAT 2.9.3 (Goudet, 1995). When P was < 0.05, significance levels were adjusted with the sequential Bonferroni correction for multiple tests (Rice, 1989).

Effect of matrix on genetic subdivision and genetic diversity

I pooled pairwise F_{ST} estimates from within the two tussock and two pasture sites and used a two-sample randomisation test (resampled without replacement, 9999 iterations) implemented in POPTOOLS 2.5.3 (Hood, 2002) to test whether the mean pairwise F_{ST} was lower between rocks in pasture than tussock. I also tested whether populations in pasture matrix had lower genetic diversity than those in tussock based on two measures

of genetic diversity: (1) allelic richness, a measure of the number of alleles in a population corrected for sample size (Petit *et al.*, 1998), and (2) gene diversity (Nei, 1987). These were pooled from the tussock and pasture sites and one-sided tests were performed using the permutation procedures in FSTAT with 1000 permutations.

Isolation by distance

I used a test for isolation by distance to determine the spatial scale at which skink dispersal was restricted. This was conducted with a mantel test (Mantel, 1967) implemented in POPTOOLS to test for a significant correlation between genetic differentiation between pairs of rock outcrops (F_{ST}) and distance. I applied the test to data transformed according to Rousset (1997), who showed that in a two-dimensional landscape a linear relationship is expected between $F_{ST}/(1-F_{ST})$ and Ln distance. I used Pearson's correlation coefficient, r, as the test statistic and 9999 randomisations of the distance matrices. I performed this test for all pairwise comparisons and for pairs of populations within sites only. Minimum distances between rocks were estimated using a Garmin GPS (± 5 m accuracy) and 50 m measuring tapes. Tests for isolation by distance were not conducted within each of the matrix types because of small sample sizes.

Sex-biased dispersal

Grand skinks have four easily distinguished age classes (≤ 1 year, ≤ 2 year, ≤ 3 year, adult; Whitaker, 1996), and adults can be sexed by the presence of subcutaneous hemipenes in males. I tested for sex-biased dispersal by comparing the mean corrected assignment index of adult males and females (mAIc; Favre *et al.*, 1997), and also F_{ST} between adult males and females. The tests were implemented in FSTAT 2.9.3 and tested for significance with a permutation procedure where the test statistic (t-statistic; Goudet *et al.*, 2002) was compared to a null distribution of the statistic generated by randomising gender with respect to genotype 10,000 times, and recalculating the statistic. I carried out separate tests for populations in tussock (six rock outcrops) and pasture (six rock outcrops). Two-sided tests were used because there was no a priori expectation for one sex to disperse more than the other.

Analyses of individual multilocus genotypes

I estimated the probability that individual skinks were born on each of the candidate rocks at each site with a Bayesian-model-based clustering approach implemented with

the program STRUCTURE 2.0 (Pritchard *et al.*, 2000). Structure allows prior information to be incorporated into the model, and to aid clustering I used mark-recapture records to determine the natal rock of a subset of the skinks under study (131 of 261 individuals). These individuals had been studied as part of a long-term mark-recapture investigation (M. Tocher pers. comm.; O. Berry unpubl. data). In the model, I specified that I had high confidence in the natal rock outcrop of these skinks but allowed a small probability ($\nu = 0.03$) that these classifications were wrong (0.03 is the empirically determined error rate expected from my definition of natal rock, see Chapter Three for details). I used this prior information to foster clustering of the remaining individuals whose natal rocks were unknown. The STRUCTURE program estimates the posterior probability (Q) that each skink was born on each of K candidate rock outcrops at each site. Because the existing mark-recapture data showed that few individuals (3 of 131) originated from outside the study sites (M. Tocher pers. comm.), I specified K to equal the number of rocks at the site. I also specified that allele frequencies on different candidate rock outcrops were correlated (FREQSCORR = 1) and left the remainder of the input parameters at default values. I ran the clustering for a burnin of 50,000 iterations followed by a run for 1×10^5 iterations of the Markov chain. I ran ten replicates for each site to check for multimodality in the clustering process.

Results

Sample and population sizes

A total of 261 skinks were genotyped from the study rocks. The number of skinks genotyped was very close to estimated population sizes on five rocks (Table 1). Because capture effort was more than twice as high at rocks where population estimates were not made, it is likely I genotyped nearly all the skinks inhabiting each rock outcrop.

Characteristics of microsatellite markers

The 15 microsatellite loci typed were highly variable. The average number of alleles per locus was 15.73 (± 1.16 SE), and the average observed heterozygosity was 0.76 (± 0.03 SE). Populations on each rock outcrop also exhibited high genetic diversity (Table 1). Based on a global test, there was no significant overall departure from Hardy-Weinberg equilibrium ($P > 0.05$). However, tests per locus and per rock outcrop showed that two loci had significant deficits of heterozygotes, indicating the existence of null alleles on some rock outcrops. I excluded these loci from the remaining analyses. Loci were not significantly in linkage disequilibrium.

Genetic subdivision

Skink populations were significantly genetically structured. The overall level of genetic subdivision (F_{ST}) among all 12 rock outcrops sampled was 0.097 ± 0.006 SE, which was significantly different from zero ($P < 0.0002$). Furthermore, all pairwise tests for differentiation between rock outcrops were significant except between rocks P1.1 and P1.2.

Effect of matrix type on genetic subdivision and genetic diversity

The F_{ST} was lower at the two tussock dominated sites than the two pasture dominated sites (Figure 2). In addition, the mean pairwise F_{ST} at tussock-dominated sites was significantly lower than at pasture-dominated sites ($P = 0.03$), and both allelic richness and gene diversity were significantly lower on rocks in pasture than in tussock ($P < 0.05$; Figure 3).

Table 1. Summary statistics for 12 grand skink populations genotyped for 13 microsatellite loci.

Site	Rock	Distance to nearest rock (m)	Population estimate[a]	Minimum population index[b]	*n* genotyped[c]	A[d]	H_{exp}[e]	H_{obs}[f]
P1	1	71	13.09 (8.85-25.13)	12	12.0 ± 0.0	6.5 ± 0.4	0.787 ± 0.004	0.769 ± 0.123
	2	71	-	14	12.8 ± 0.2	5.8 ± 0.7	0.744 ± 0.007	0.669 ± 0.236
	3	164	-	38	37.4 ± 0.3	9.0 ± 0.8	0.776 ± 0.020	0.803 ± 0.108
T1	1	61	-	32	22.7 ± 0.4	8.2 ± 0.7	0.793 ± 0.045	0.800 ± 0.180
	2	34	-	22	21.1 ± 0.3	8.1 ± 0.8	0.794 ± 0.022	0.816 ± 0.082
	3	89	-	25	22.0 ± 0.0	8.4 ± 0.6	0.800 ± 0.040	0.860 ± 0.178
	4	34	-	16	15.9 ± 0.1	8.2 ± 0.7	0.800 ± 0.042	0.820 ± 0.193
P2	1	223	28.24 (23.17-36.15)	29	28.6 ± 0.2	7.9 ± 0.5	0.780 ± 0.026	0.834 ± 0.122
	2	184	24.24 (19.45-32.17)	27	26.4 ± 0.1	6.8 ± 0.4	0.728 ± 0.051	0.766 ± 0.214
	3	184	17.57 (13.36-25.65)	19	18.3 ± 0.2	6.2 ± 0.0	0.741 ± 0.045	0.791 ± 0.188
T2	1	135	22.88 (20.74-25.51)	24	22.0 ± 0.7	7.5 ± 0.5	0.768 ± 0.045	0.811 ± 0.176
	2	135	-	15	15.0 ± 0.0	6.8 ± 0.4	0.760 ± 0.050	0.744 ± 0.207

[a]Population estimate = mark-recapture estimate of population size (95% CI). - indicates a mark-recapture estimate was not made. [b]Minimum population index = the total number of skinks captured on a rock outcrop. [c]*n* genotyped = average number of skinks genotyped at each locus, [d]A = average number of alleles at each locus, [e]H_{exp} = average gene diversity at each locus (Nei 1987), [f]H_{obs} = average observed heterozygosity at each locus. All estimates are ± SE.

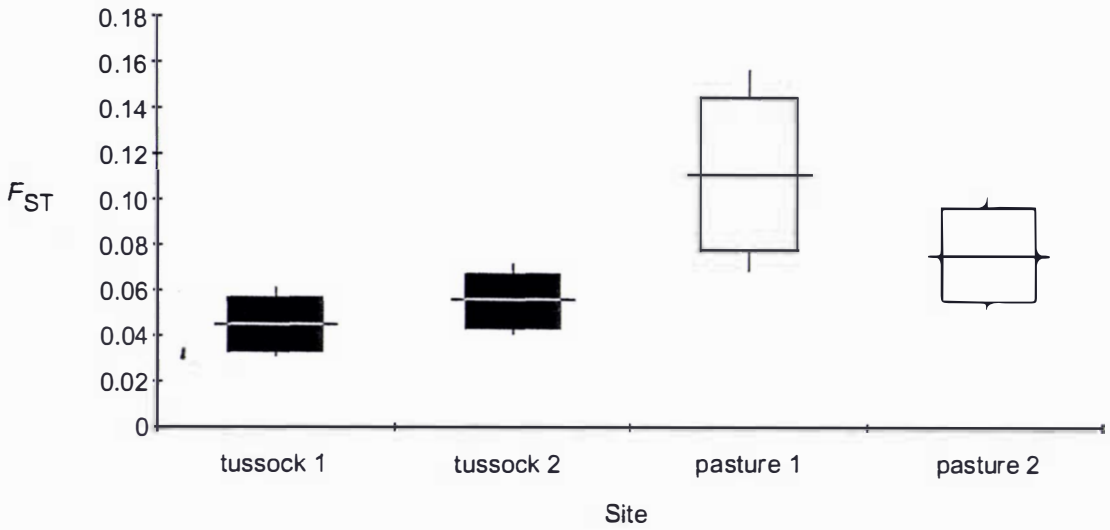

Figure 2. Comparison of genetic differentiation (F_{ST}) between rock outcrops at each study site (horizontal line), and showing 95 and 99% CI derived by bootstrapping over loci (box and whiskers respectively)

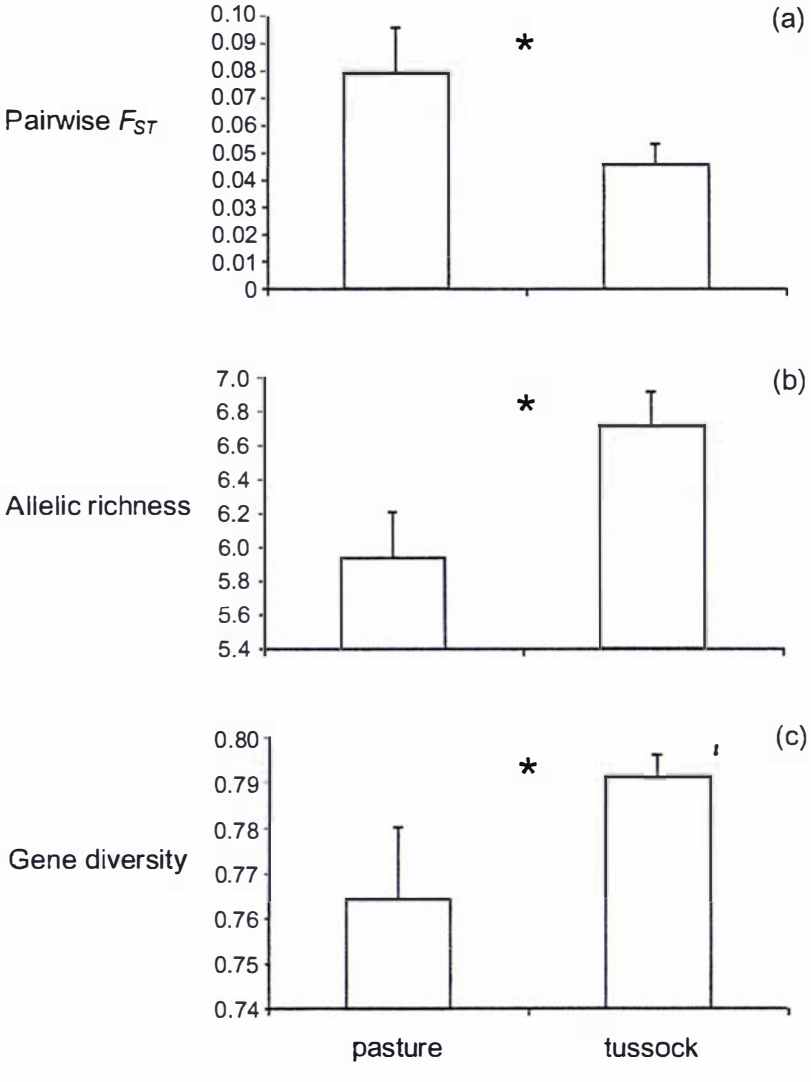

Figure 3. Comparison of (a) mean pairwise F_{ST}, (b) allelic richness, and (c) gene diversity in pasture and tussock dominated sites (with SE). An asterisk (*) indicates a significant difference ($P < 0.05$).

Isolation by distance and sex-biased dispersal

There was a positive and significant association between genetic differentiation (F_{ST}/1-F_{ST}) and Ln distance between rock outcrops both overall and within sites (overall r = 0.669, $P < 0.001$; within sites $r = 0.502$, $P = 0.041$; Figure 4).

In tussock dominated sites, the test based on mean corrected assignment index detected significant female-biased dispersal (male mAIc = 1.52, n = 15, female mAIc = -1.14, n = 20, P = 0.05), but no evidence for sex-biased dispersal was detected among the populations in pasture (male mAIc = -0.638, n = 21, female mAIc = 0.216, n = 26, P = 0.46). Similarly, tests based on F_{ST} detected significant female biased dispersal in tussock (male F_{ST} = 0.089, female F_{ST} = 0.006, P = 0.02), but not in pasture (male F_{ST} = 0.069, female F_{ST} = 0.113, P = 0.23).

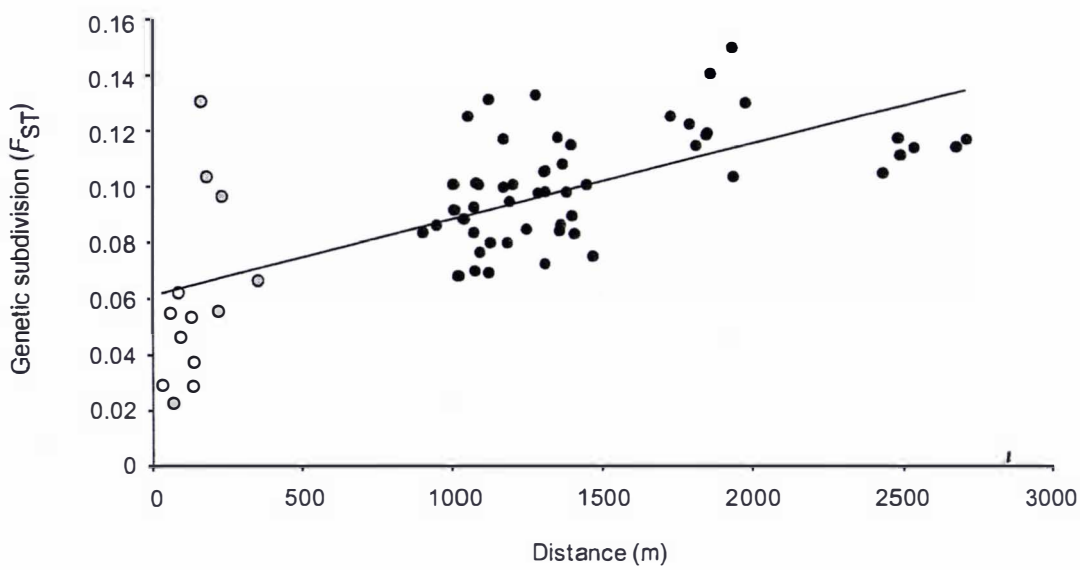

Figure 4. The relationship between genetic differentiation (F_{ST}) and distance between skink populations. Open circles indicate pairwise F_{ST} between rocks within study sites in tussock, grey circles indicate pairwise F_{ST} between rocks within study sites in pasture, and closed circles indicate pairwise F_{ST} between rocks from different study sites.

Analyses of individual multilocus genotypes

Examination of the range of values of Q for individuals in tussock and pasture (Figure 5) revealed patterns of dispersal that were sometimes not obvious from population-level analyses illustrated in Figures 2 and 3. At pasture site P2 seven skinks had higher probabilities of belonging to rocks other than their capture rocks, meaning that these individuals were possible dispersers. These assignments are strongly supported because Chapter Three showed that assignments of skinks at this site were 100% correct relative to known mark-recapture data.

Clustering of the data for pasture site P1 converged at two different modes in replicate runs, indicating that the loci did not have sufficient power to consistently reveal population structure. This multimodality occurred because of the failure to resolve genetic structure between rocks P1.1 and P1.2, which lacked significant genetic structure and were effectively a single population. Combining these populations and rerunning the clustering consistently resulted in a single mode. In this case only five individuals had probabilities of originating on rock outcrops different from their capture rock (Figure 5). These individuals are likely to be dispersers because Chapter Two showed that assignments of skinks at this site relative to mark-recapture data were accurate in 95% of cases.

More complex patterns were evident for the two tussock sites, with some rock outcrops appearing to be highly admixed, whereas others showed little admixture (Figure 5). At tussock site T2 10 skinks from rock T2.1 had high probabilities of belonging to rock T2.2, but no skinks from rock T2.2 had high probabilities of belonging to rock T2.1. Similarly, at tussock site T1 few skinks from either rocks T1.1 or T1.3 had high probabilities of belonging to another rock, but many skinks from rocks T1.2 and T1.3 had high probabilities of belonging to other rock outcrops. In the case of rock T1.2, most of these individuals were assigned to rock T1.3, and in the case of rock T1.3, most individuals were assigned to rock T1.1. Most of these skinks are likely to be dispersers because Chapter Two showed that between 79% (site T1) and 65% (site T2) of skinks were assigned correctly relative to mark-recapture data at these sites.

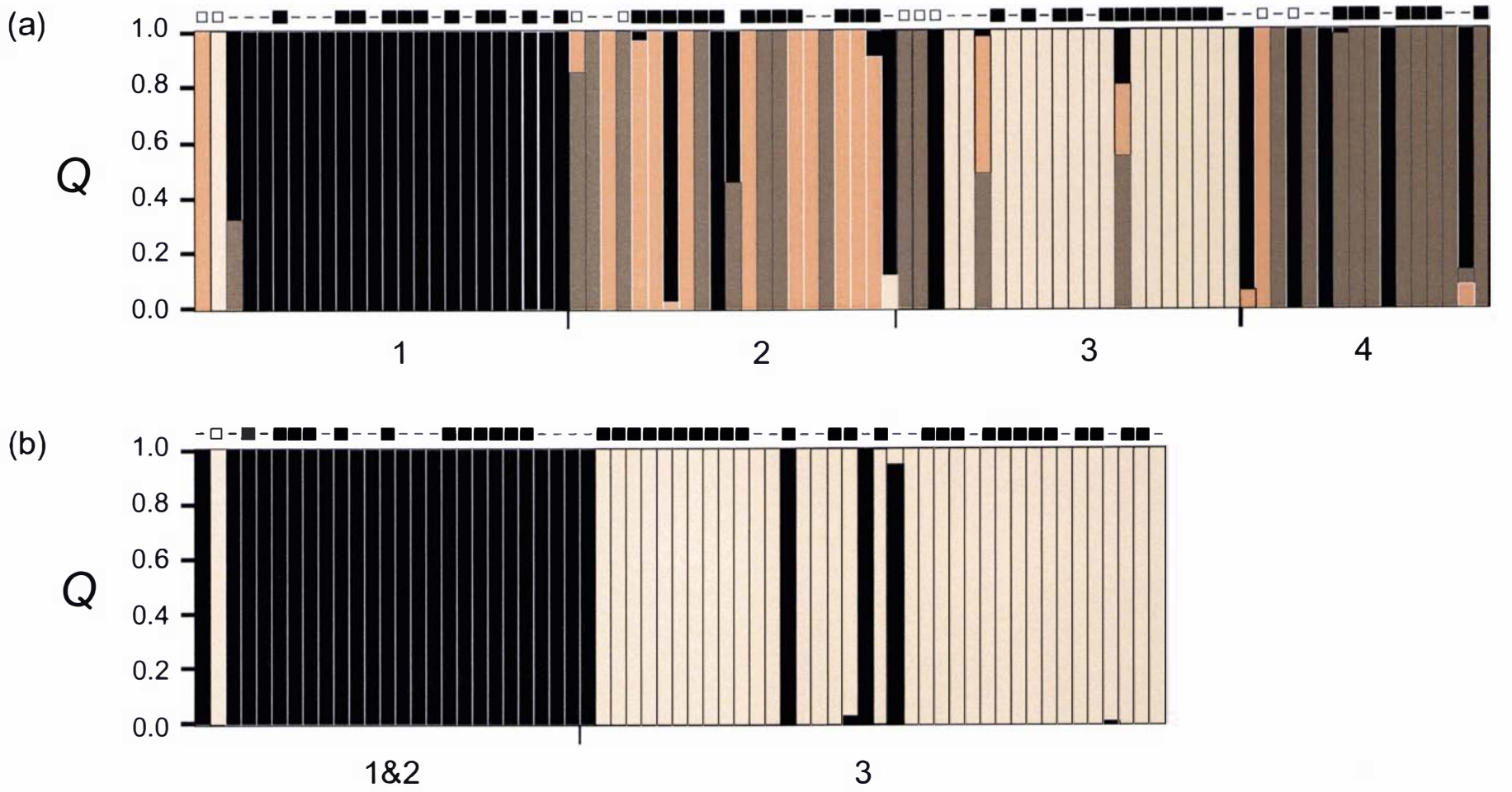

Figure 5. Results of Bayesian clustering of individual skink genotypes with STRUCTURE 2.0. Each site is shown separately: (a) site T1, (b) site P1, (c) site T2, (d) site P2. Individuals are represented across the x axis by a vertical bar that may be divided into shaded segments that represent that individual's probability of originating (*Q*) from each of the rocks at a study site. Skinks are also grouped across the x axis according to the rock they were captured on, (continued).

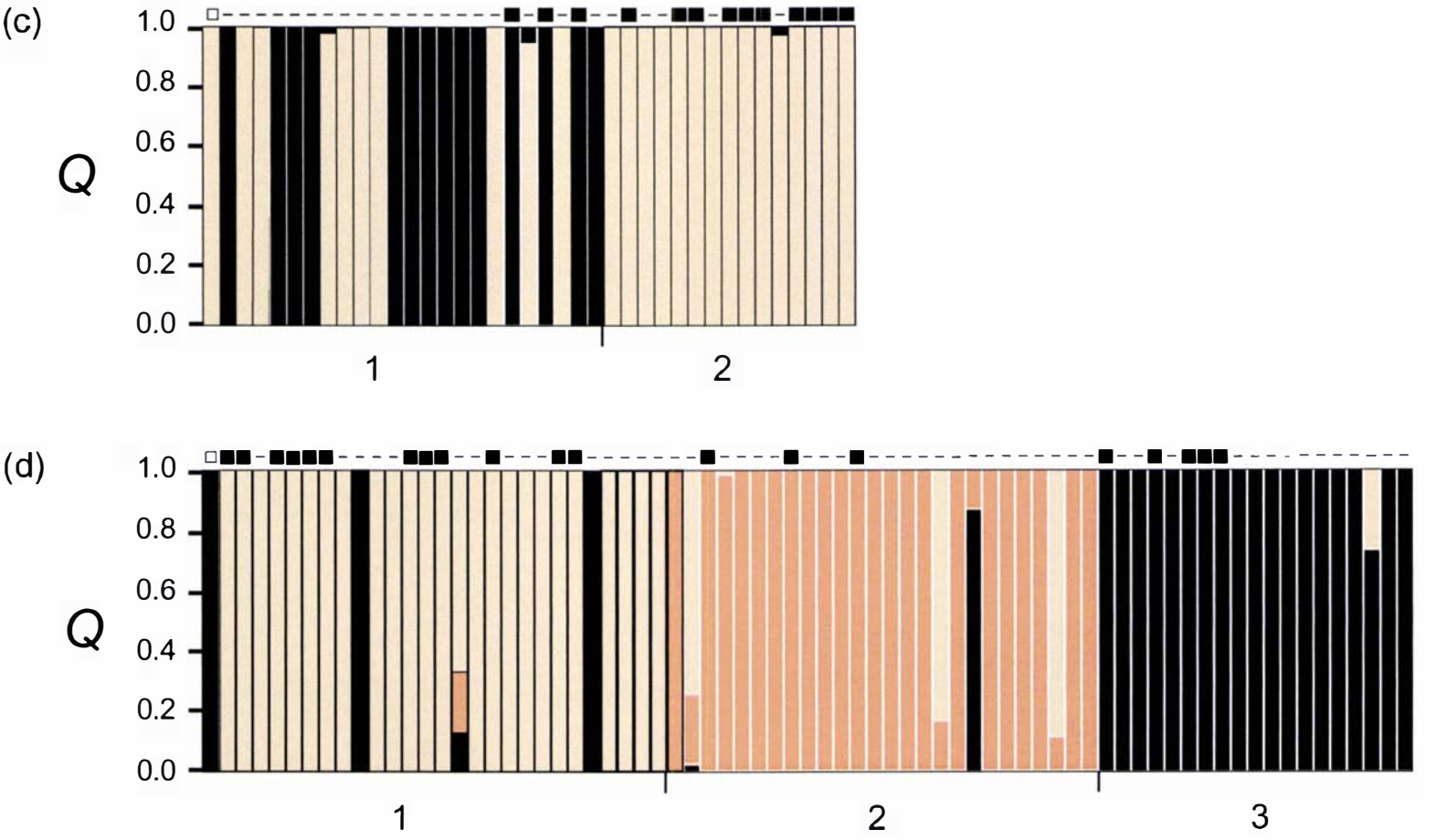

Figure 5. (continued) and skinks assigned to each population are shaded a different colour. Filled squares above an individual indicate that the natal rock was known and the individual did not disperse. Open squares indicate that the individual was a known disperser. Dashes indicate that the natal rock was not known for that individual

Discussion

The global spread of intensive agriculture means that many species now occur as small populations embedded within highly modified landscapes. Although corridors are a widely accepted approach to connecting populations (Rosenberg *et al.*, 1997), their merits are still debated (Beier & Noss, 1998), and they are likely to be impractical in many situations. A focus on managing the matrix has been advocated as an alternative way to deal with habitat fragmentation (Perfecto & Vandermeer, 2002), and has theoretical support. I sought to provide an empirical account of how two forms of agricultural landuse affect the connectivity of populations of endangered skinks.

Study design and interpretation

Making inferences about landscape connectivity from genetic data poses several difficulties that must be addressed by appropriate experimental design (Steinberg & Jordan, 1998). First, one must assume that the underlying patterns of genetic variation before land-use change or fragmentation were homogenous across the study area and are not the result of historical effects such as range changes (*cf.* Cunningham & Moritz, 1998). In this case, because of the small spatial scale of the study and interspersion of replicate sites, the observed differences are unlikely to be due to historical effects.

Second, estimators of genetic structure and genetic variation can be biased by small sample size or non-random sampling (Hansen *et al.*, 1997; Mossman & Waser, 2001; Tallmon *et al.*, 2002), meaning that samples do not adequately represent the genetic variation present in populations. I used mark-recapture population estimates to show that genetic sampling of the populations was nearly complete, thus allowing me to examine the effects of the matrix with little sampling error.

Third, it is important to match the characteristics of populations in the two types of landscapes because the rate of loss of genetic variation within populations and increase in genetic divergence between populations may result from three factors: differences in the size of populations, the rate of migration, and the rate of mutation (Wright, 1969). Because only the rate of migration is of interest, the other two characters must be matched. A central feature of the design of this study was that populations in pasture and tussock were replicated and about the same size (based on minimum population size, t test, $P > 0.05$), making any observed treatment effects the result of dispersal rate (I also assumed that mutation rate is equal in pasture and tussock

populations). Although I used replicate sites in an attempt to account for inherent differences that could confound the results, population in pasture were on average farther apart than those in tussock (205 m ± 38.3 SE *vs.* 92 m ± 20.7 SE). This should increase genetic subdivision in pasture relative to tussock, and without additional study sites I could not determine the extent to which this contributes to my results. However, this difference was largely due to widely separated populations at pasture site P2 because site P1, which had been pasture for the longest period (over 18 years), had the most genetically subdivided populations. Yet, its populations were not separated by distances significantly different from those in tussock (*t* test, $P > 0.05$), suggesting that it is the matrix limiting dispersal that is a more critical factor.

Environmental and genetic patchiness

Grand skinks live in a naturally patchy environment, and this patchiness is reflected in the fine-scale of the genetic structure detected. Several lines of evidence indicate that dispersal is very localized in this species. First, a pattern of isolation by distance existed at both small (< 350 m) and moderate (< 2.5 km) spatial scales, indicating that skinks disperse more often between nearby rock outcrops than more distant rock outcrops (even at scales ≤ 350 m). Second, neighbouring populations were genetically distinct in nearly all cases, even though some were only 35 m apart (e.g., between rocks T1.2 and T1.3). These observations are supported by mark-recapture data showing that some skinks to have remained on a single rock for over 12 years (M. Tocher pers. comm.).

Although the fine scale of genetic structuring indicated that dispersal was restricted between rock outcrops, it is unlikely that the high levels of genetic diversity recorded on each rock outcrop could be sustained in such small populations without recurrent dispersal (*cf.* Tallmon *et al.*, 2002). Completely isolated populations with population sizes similar to those recorded here ($n \sim 25$) would probably have effective population sizes (N_e) 10-50% of that size (Frankham, 1995) and would lose neutral genetic variation rapidly. For example, without immigration, they would on average lose 50% of their heterozygosity within 1.39 x N_e generations, which is between 3.5 and 17.4 generations (Hartl & Clark, 1997). The high levels of genetic variation observed indicates that despite the patchiness of the rocky habitat, regular short-distance dispersal must also be a characteristic of this species. This provides independent evidence that grand skinks persist in this patchy environment through the formation of a metapopulation (Whitaker, 1996). Further, it makes focus on dispersal particularly

relevant in this species because species with limited dispersal abilities may be more responsive to the nature of the matrix than more vagile species (Ricketts, 2001).

Importance of matrix

Theory predicts that small, isolated populations are more subject to genetic drift than populations experiencing dispersal and gene flow, should feature less genetic variation, and should be more genetically structured (Wright, 1969). The results of this study are consistent with these expectations – populations in pasture were predicted to be more insular than those in tussock (Whitaker, 1996), and I showed that they had less genetic variation and were more genetically structured than similar populations in tussock. This result is notable because results from short-term ($\leq$ 2 years) studies of dispersal based on mark-recapture data have not shown any significant effect of the matrix on rates of dispersal (Whitaker, 1996; Houghton, 2000). Importantly, reduced dispersal in pasture-dominated landscapes means that populations should be more subject to stochastic processes (Hanski, 2001), and explains why populations are patchier and more extinction prone (Whitaker, 1996).

Skink sources and sinks? – Individual-based analysis

Individual-based genetic analyses revealed that patterns of dispersal relative to the vegetation matrix were more complex than indicated by standard qualitative genetics analyses. Based on the qualitative genetic results from tussock study sites, I expected more skinks to be identified as likely dispersers than in the pasture sites. Yet, I observed that some rock outcrops were highly admixed as expected, whereas others were relatively insular. For example, rocks T1.1 and T2.2 both received a few or no immigrants but contributed many to neighbouring rock outcrops. It is unclear whether this patterns is indicative of source-sink dynamics (Harrison, 1991), or what features of these rock outcrops promote greater emigration than immigration, but density-dependent emigration resulting from resource limitation may merit investigation (e.g. Gaggiotti *et al.*, 2002).

The assignment results from the pasture sites were more in line with the expectations from the qualitative genetic analysis, with few skinks from any rocks identified as likely dispersers. The one exception was between two rocks, which were highly admixed, at site P1. These populations were separated by 71 m, but two small

rock outcrops inhabited by a handful of skinks (≤ 3) were situated in the intervening space and may have facilitated the high level of dispersal by acting as stepping stones.

Sex-biased dispersal and the matrix

Sex-biased dispersal has been identified in a handful of lizard species and in most cases has been male biased (e.g. Doughty *et al.*, 1994; Stow *et al.*, 2001). My results show that (1) neither sex is strictly philopatric because both male and female individuals had low mAIc scores (data not shown), (2) female-biased dispersal was a feature of populations in tussock, and (3) sex-bias was absent in pasture. The first observation is supported by data from a mark-and-recapture study that detected both male and female dispersers between rock outcrops (M. Tocher pers. comm.; Houghton, 2000). The effect of the matrix on sex-biased dispersal (observations 2 and 3) is important because it indicates that not only did the pasture matrix reduce levels of dispersal, but also it may have qualitatively affected the individuals that disperse. This effect of habitat fragmentation is seldom studied (see Stow *et al.*, 2001), and it is unclear whether it might detrimentally alter the demography or reproductive behaviour of isolated populations. The detection of a sex bias in dispersal is also notable because no consistent sex bias in interpopulation dispersal was detected in earlier mark-recapture studies of this species (Whitaker, 1996; Marshall, 2000; Houghton, 2000).

Implications for skink and tussock grassland conservation

Most studies of habitat fragmentation focus on forest-dwelling mammals or birds and rarely on reptiles or grasslands (McGarigal & Cushman, 2002). Yet in some cases these groups represent major components of regional biodiversity. In New Zealand, where native terrestrial mammals are absent, reptiles form a large proportion of vertebrate biodiversity, but most species have suffered major range declines or extinctions since human settlement (Towns *et al.*, 2001). Similarly, montane tussock grasslands were historically a prominent vegetation assemblage in southern New Zealand but have been greatly reduced in area by agricultural development (McGlone *et al.*, 1995; McGlone, 2001). The results presented have relevance to the conservation of both the grand skink and native tussock grasslands. The grand skink is thought to have suffered a massive range collapse (Whitaker & Loh, 1995; see Chapter 6). Because this coincided with the widespread loss of native vegetation it has been speculated that the processes are related (Whitaker & Loh, 1995; Whitaker, 1996). I have demonstrated an effect of matrix vegetation that has the potential to alter landscape connectivity for grand skink

populations and possibly even alter social dynamics. This is cause for concern because it is well established that small populations are vulnerable to stochastic extinctions due to both demographic and genetic causes should they become isolated (Lande, 1988). However, linking landscape change to the decline of this species is complicated by the simultaneous pressures of multiple introduced mammalian predators (Towns *et al.*, 2001), which may act in combination with landscape change to endanger skink populations. The effects of introduced predators on skink populations are currently under study (M. Tocher pers. comm.).

Summary

I aimed to provide an empirical example of how different agricultural land uses affect connectivity of animal populations. The combined genetic and demographic data enabled me to make four main observations about the significance of vegetation matrix for skink dispersal: (1) grand skinks live in small groups on rock outcrops and usually disperse short distances; (2) on average dispersal between populations in pasture was less frequent than between populations in tussock; (3) female skinks were more likely to disperse than males between rock outcrops in tussock but not in pasture; and (4) despite (2), not all rocks in tussock were characterized by high emigration and immigration – some appeared to function as sources of immigrants to neighbouring populations and received relatively few immigrants themselves. Together, these results suggest that the nature of the matrix can both quantitatively and qualitatively affect dispersal, but characteristics of individual populations may also play a role in determining dispersal dynamics and warrant further attention in studies of habitat fragmentation. Managing the vegetation matrix may be a practical way to connect fragmented populations in some situations.

Acknowledgements

I thank Mandy Tocher for sharing her mark-recapture data with me and Jeroen Spitzen, Annemarieke van der Sluijs, and Amanda Smale for assistance with skink catching and good humour. Dave Houston and Graeme Loh provided valuable skink-related discussions, and Sam Banks and two anonymous referees provided comments that improved the manuscript.

5

Inbreeding and promiscuity in the endangered grand skink

Abstract

The inbreeding avoidance hypothesis predicts that organisms that often encounter relatives as potential mates should evolve behaviours to avoid incestuous matings. Avoidance behaviours have practical importance for small populations because deleterious genetic processes may be less imminent than otherwise expected from genetic models that assume random mating. I used genetic techniques to investigate the extent of inbreeding and inbreeding avoidance behaviours in rare lizards from southern New Zealand. Grand skinks, *Oligosoma grande*, live in small patchily distributed groups, and have low rates of inter-group dispersal (*ca.* 3-20% disperse). I used data from 15 microsatellite loci to test the hypothesis that adults are likely to encounter kin as potential mates and will inbreed. These data confirmed that adult skinks usually inhabited rock outcrops with adult relatives of the opposite sex - up to 35% of potential mates were of equivalent relatedness as half-sibs and 17% were equivalent to full sibs. However, skinks did not preferentially breed with less related mates, and 18.2% of matings were between individuals of equivalent relatedness as full-sibs. Instead, skinks mated with partners of all levels of relatedness, and were promiscuous - almost half of adult females and nearly three quarters of adult males reproduced with multiple partners. In addition, inbreeding had no effect on survival of offspring in their first year. Two other putative mechanisms of inbreeding avoidance, sex-biased and natal dispersal, were not pronounced in this species. This study adds to a growing list of species that inbreed despite the risks.

Introduction

Addressing the dual threats of inbreeding depression and loss of genetic variation is central to many conservation programs, particularly those focussed on small or captive populations (Caughley, 1994). These concerns stem mainly from theoretical, laboratory and captive animal studies suggesting that inbreeding can reduce individual fitness (Lynch *et al.*, 1995; Frankham, 1995), and that populations with low genetic variation may be unable to respond adaptively to environmental change (Fisher, 1930; Lande & Shannon, 1996; Franklin & Frankham, 1998).

The extent of inbreeding depression in the wild is less well documented and remains controversial (Frankham, 2001; Elgar & Clode, 2001). In part this is because of the inherent difficulty of demonstrating inbreeding effects in the wild (Frankham & Ralls, 1998), but it may also reflect a genuine pattern because wild animals may have mechanisms to avoid mating with close relatives (the inbreeding avoidance hypothesis, Ralls *et al.*, 1986; Harvey & Ralls, 1986; Waldman & McKinnon, 1993; Pusey & Wolf, 1996). Two putative examples of such mechanisms are kin recognition and avoidance (Waldman *et al.*, 1992), and natal or sex-biased dispersal (Johnson & Gaines, 1990). These behaviours have practical implications for the management of small populations, such as those affected by habitat fragmentation, because inbreeding depression and loss of genetic variation will occur more slowly than predicted from population genetic models that assume random mating (Falconer, 1989; Frankham, 1995; Stow & Sunnucks, 2004; Earnhardt *et al.*, 2004; but see Montgomery *et al.*, 2000).

Empirical evidence for inbreeding avoidance in animals has been mixed and comes mostly from mammals and birds (e.g. Cockburn *et al.*, 1985; Wheelwright & Mauck, 1998; Keller & Arcese, 1998; Duarte *et al.*, 2003), and its prevalence in the wild has been debated (Ralls *et al.*, 1986; Shields, 1993; Komdeur & Deerenberg, 1997). Some of the contention stems from the difficulty of obtaining accurate data on dispersal and mating systems by observation (Koenig *et al.*, 1996; Parker & Waite, 1997); a problem which has now been largely overcome with the development and application of highly variable co-dominant DNA markers, which permit detailed analyses of mating systems, kinship (Queller *et al.*, 1993), and natal and sex-biased dispersal (Favre et al., 1997). These approaches provide an opportunity to clarify the extent of inbreeding, inbreeding depression, and inbreeding avoidance in wild populations. Further, this understanding will be strengthened by a focus on non-

mammalian and non-avian animal groups, which have been underrepresented in studies to date (Waldman & McKinnon, 1993; but see Olsson et al., 1996b; Stow & Sunnucks, 2004).

A prerequisite for the evolution of inbreeding avoidance is the opportunity for incestuous matings (Ralls et al., 1986; Pusey & Wolf, 1996). Small and insular populations should therefore be good models for investigation, and here I investigate the mating system, patterns of kinship, dispersal, and juvenile survival in small insular populations of an endangered lizard from southern New Zealand. The grand skink, *Oligosoma grande*, lives in small groups of *ca.* 20 individuals on house-sized rock outcrops that are separated from similar groups by 50-150 metres of inhospitable vegetation matrix. Previous work has shown that dispersal between rock outcrops is limited on small spatial scales (35-350 metres, Chapters 3 and 4), which leads to the prediction that adults will share outcrops with kin and potentially inbreed. Further, habitat modification has increased the isolation of some skink populations (Chapter 4), increasing the likelihood that deleterious genetic processes will occur (Frankham et al., 2002).

I used data from 15 microsatellite loci and parentage analysis to examine four questions related to inbreeding and inbreeding avoidance in the grand skink. First, I investigated whether grand skinks have opportunities to mate with close relatives (equivalent to half or full sibs). Second, I tested whether skinks avoid breeding with close relatives. Third, I examined evidence for avoidance of inbreeding by natal or sex-biased dispersal. Finally, I tested whether the offspring of less related parents had higher survivorship in their first year of life than the offspring of more related parents. In sum, I ask whether inbreeding avoidance behaviours will mitigate against the deleterious genetic effects of isolation caused by habitat fragmentation.

Methods

Field methods

The study area was located at "Redbank", near Macraes Flat, southern New Zealand (45° 25'S, 170° 24'E; Figure 1). Part of this area is a reserve for lizard conservation and consists of a mosaic of tussock grassland and agricultural pasture regularly punctuated by house-sized schist rock outcrops. Nylon fishing line nooses were used to capture skinks from two pasture-dominated (P1 & P2) and two tussock-dominated sites (T1 & T2). Skinks were measured, marked with a unique toe-code, and adults were sexed before being released at their point of capture. Nearly all resident skinks on each outcrop were captured by repeatedly visiting rock outcrops over their entire active period (November – May). This was verified by estimation of the population sizes by mark-recapture analyses (Chapter 4). In addition, the skink populations have been monitored intensively since 1995 (Whitaker, 1996; M. Tocher, pers. comm.).

Laboratory analysis

Tissue samples from all captured skinks were collected for genetic analysis. Samples consisted of toe-clips or tail-tips (< 2 mg tissue), and were placed immediately into liquid nitrogen. DNA was extracted by a salting-out procedure (Sunnucks & Hales, 1996). A total of 261 skinks were genotyped for 15 microsatellite DNA loci following the methods described in Chapter Two.

Parentage

The parentage of the two cohorts of skinks born in the late summer of 2000 and of 2001 was determined using a likelihood approach implemented by the program CERVUS 2.0 (Marshall *et al.*, 1998). Almost a year elapses between mating and birth in this species (Cree, 1994), and the offspring studied were the result of matings that occurred in Autumn (Feb-April) 1999 and 2000 respectively. Because detailed capture-recapture history was known for skinks from 1995 on (M. Tocher pers. comm.), I could accurately determine the number of candidate parents for each offspring, and also the number that had not been genotyped (because they were not captured in the 2000/2001 season). Parentage was assigned in a stepwise manner, firstly assigning females, then males using the prior parentage information where confidence in maternity was ≥ 0.95. If maternity could not be assigned with ≥ 0.95 confidence, paternity was assigned

without the maternal information. The parameters used for simulations differed between study sites and are listed in Appendix 5.1.

Grand skinks have four easily recognisable age/size classes based on their snout-vent length: ≤ 1 year old, ≤ 2 year old, ≤ 3 year old, and adults, and are believed to become sexually mature in their fourth year (Cree, 1994; Whitaker, 1996), and therefore I included all skinks of four years or older as candidate parents. While no female skinks in their third year have been recorded as gravid (M. Tocher pers. comm.), I could not discount males of this age being potential fathers. To allow for this possibility I included three-year-old males as candidates. CERVUS uses simulations of parentage to establish confidence in parental assignments, and requires empirical allele frequencies. I used site-wide allele frequencies for these simulations, and although in Chapters Three and Four I found significant genetic subdivision among rock outcrops at each study site, the effect of pooling allele frequencies from all rocks at each site had little effect on statistical confidence (analyses not presented).

Kinship on rock outcrops

I used the program KINSHIP 1.1 (Goodnight & Queller, 1999) to estimate pairwise relatedness between all adult skinks (R) using the method of Queller and Goodnight (1989). Because inclusion of many related individuals in the population causes downwards bias of relatedness among relatives, I removed all ≤ 2 year old skinks from relatedness calculations.

I used two approaches to determine the proportion of adult opposite sex pairs that were related. First, I used KINSHIP to simulate 1000 unrelated pairs of skinks from allele frequencies at each study site and calculated the 95% confidence limits on R values between these pairs. Using this information I estimated the chance of a skink mating with kin as the proportion of all opposite sex pairs on each rock outcrop that had R values greater than the upper 95% confidence limit for unrelated skinks. Second, I tested the hypotheses that each potential pair involved either half or full sibs (H_a) against the null hypothesis that pairs were either unrelated or half-sibs respectively (H_o). KINSHIP was used to calculate the likelihood ratio of H_a and H_o based on the allele frequencies at each study site. A test for the significance of H_a was conducted by simulating 1000 pairs of individuals according to the H_a and H_o hypotheses and estimating directly the ratio needed to reject H_o (Goodnight & Queller, 1999). The

likelihood ratio tests provide more power to detect pedigree relationships than simply assessing the R value relative to 95% confidence intervals of unrelated skinks from simulation. Thus, in some cases likelihood ratio tests are able to achieve significance where the observed R value of a pair was within the simulated 95% confidence limits for unrelated skinks.

Kinship in tussock and pasture

Grand skink populations occur in both modified (pasture) and unmodified (tussock grassland) landscapes. I used a two-sample randomisation test implemented in POPTOOLS (Hood, 2002) to test whether skinks on rocks in pasture had a higher percentage of close kin (half-sib or greater) as potential mates than skinks on rocks in tussock.

Breeding partners

I tested whether male or female skinks reproduced assortatively according to relatedness by comparing R for the mated pair with the average R of each of the mated individuals to all non-mated opposite sex candidates on the same rock outcrop. The test was performed by randomisation, where the test statistic was a paired t-value from the observed data, which was compared to the distribution of the same statistic calculated for 9999 randomised datasets. These comparisons assume that all individuals were available to mate. I have no data for males, however, on average, 90.3% of females breed each year (Cree, 1994).

Offspring survival

As a test of whether offspring of more inbred matings had lower survival, I compared the relatedness (R) of parents of offspring that were captured just after birth with the relatedness of those that were caught approximately a year after birth using a two-tailed t-test. In addition, because it allowed inclusion of offspring for which I could assign only one parent (and hence provides greater power), I compared the internal relatedness (IR, Amos *et al.*, 2001) of offspring from these two cohorts. Internal relatedness is a modification of the Queller and Goodnight (1989) relatedness estimator, but is calculated for individuals. It is a measure of the genetic correlation between alleles within an individual, weighted by the frequency of those alleles in the population. I also tested for a difference in the variance of IR and R between newborn and one year old skinks using a two-tailed variance ratio test (Zar, 1996).

Sex-biased dispersal

I tested for sex-biased dispersal in both tussock and pasture and overall by comparing mean relatedness among adult males and adult females on each rock outcrop with two-tailed paired t-tests.

Results

Genotypic data

In total, 261 skinks were genotyped. The loci were highly variable, with a mean observed heterozygosity of 0.77 (± 0.04 SE) and an average number of alleles per locus per rock outcrop of 7.4 (± 0.30 SE). Additional descriptive statistics are provided in Chapters Two and Three. Overall, the loci were in Hardy-Weinberg equilibrium and not in linkage disequilibrium, however two loci, Oligr2 and Oligr15 had significant deficits of heterozygotes on some rock outcrops, consistent with the presence of null alleles (Chapter 2). These loci were only included in analyses at study sites where they were in Hardy-Weinberg equilibrium (see Appendix 5.1).

Parentage analysis

The loci used had very high exclusionary power (≥ 0.999 exclusionary power of the first parent). I examined the parentage of a total of 63 offspring from three study sites (29 in the 1999/2000 cohort and 34 in the 2000/2001 cohort). Study site T2 was not included in parentage analysis because only two offspring were captured. The number of candidate female parents ranged between 13 and 17 at each study site and between 8 and 17 candidate males (Appendix 5.1).

Overall, 33 offspring had both parents assigned. For the second cohort of young (newborns) the mother of all but one (33/34), and the father of 24 could be assigned with ≥ 95% confidence. For the first cohort of young (one-year-olds) the mother of 22 (22/29) and the father of 17 could be assigned with ≥ 95% confidence. A further two offspring from this cohort were assigned mothers with ≥ 85% confidence, and three were assigned fathers with ≥ 91% confidence.

Mating system

Grand skinks were highly promiscuous as both polyandry and polygyny were common. Of the fifteen litters assigned to females where more than one offspring was genotyped, seven (46.7%) were sired by multiple males. A total of 10 male skinks were assigned multiple offspring from the same cohort, and of these, seven (70%) sired their offspring with multiple females.

Kinship on rock outcrops

Kinship simulations and pairwise relatedness calculations showed that adult skinks usually shared rock outcrops with kin, and in some cases more than a third of all possible opposite sex pairs would involve related skinks (Table 1). The proportion of potential pairs that would involve at least half-sibs ranged between 0 - 35 percent and averaged 14.4 percent, and the proportion that would involve full sibs ranged between 0 and 16.7 and averaged 4.1 percent (Table 1).

Table 1. Summary of the kinship of adult grand skinks on eleven rock outcrops.

Site	Rock	No. males[1]	No. females[1]	Group size[2]	*R* 95% CI unrelated	% adult M/F pairs kin[3]	% adult M/F pairs half sibs[4]	% adult M/F pairs full sibs[5]
T1	T1.1	3	5	32	-0.19 – 0.22	13.3	6.7	0
	T1.2	2	2	22		0	25.0	0
	T1.3	4	4	25		12.5	12.5	6.2
	T1.4	3	2	16		16.7	16.7	16.7
P1	P1.1	5	4	26	-0.18 – 0.22	25.0	35.0	10.0
	P1.2	8	3	38		20.8	25.0	8.3
T2	T2.1	4	2	24	-0.21 – 0.23	12.5	12.5	0
	T2.2	2	2	15		0	0	0
P2	P2.1	4	6	29	-0.19 – 0.22	8.3	8.3	0
	P2.2	5	5	27		36.0	16.0	4.0
	P2.3	4	1	19		0	0	0

[1]Numbers genotyped - repeat visits to rock outcrops and mark-recapture analysis indicate that nearly all skinks on outcrops were genotyped, see Chapter Four. [2]Minimum group size, see Chapter Four. [3]Pairs with relatedness greater than the upper 95% confidence limit from simulations of unrelated pairs. [4]Pairs with significant likelihood ratios in tests of half sib *vs.* unrelated. [5]Pairs with significant likelihood ratios in tests of full sibs *vs.* half sibs.

Kinship in tussock and pasture

There was no significant difference in the proportion of opposite sex close relatives (half-sibs or greater) on rocks in pasture and tussock as identified by likelihood ratio tests (average percentage close relatives in tussock 12.23%, pasture 16.86%, $P = 0.51$).

Breeding partners

Of the 33 offspring for which both parents were identified, 18.2% were the product of matings between skinks with relatedness equivalent to full siblings (i.e. including

parent-offspring matings), and 21.2% were the product of matings between skinks with relatedness equivalent to or greater than half-siblings (i.e. including grandparent-grandchild matings). On average, neither female nor male skinks mated with partners that were significantly less related to them than the candidates on the same rock outcrop (mated pairs average $R = 0.11$; females to unmated males average $R = 0.06$; $P = 0.88$; males to unmated females average $R = 0.10$; $P = 0.72$).

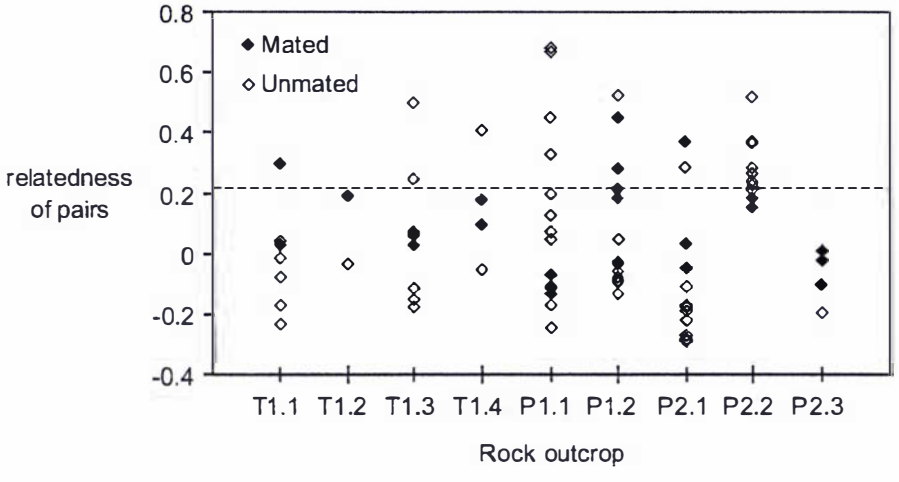

Figure 1. Relatedness values for mated (filled symbols) and potentially mated (open symbols) pairs on each rock outcrop. Potentially mated pairs include each of the known mated individuals and all it potential partners on the same rock outcrop. Dashed line indicates the upper 95% confidence limit of relatedness for unrelated pairs.

Offspring survival

Based on the relatedness of parents, offspring that survived their first year were not any less inbred than newborns (Figure 2; mean newborn $R = 0.07 \pm 0.034$ SE, mean one-year-old $R = 0.18 \pm 0.043$ SE, $t = 1.95$, d.f. = 29, $P = 0.06$), and neither was the variance in parental R any different in newborn and one-year-old skinks ($F = 1.12$, d.f. = 17,12, $P = 0.41$). Similarly, based on the internal relatedness of offspring, those that survived their first year were not less inbred than newborns (mean newborn $IR = 0.003 \pm 0.022$ SE, mean one-year-old $IR = 0.044 \pm 0.024$ SE, $t = 1.27$, d.f. = 61, $P = 0.21$), and neither was the variance in IR any different in newborn and one-year-old skinks ($F = 1.06$, d.f. = 33,28, $P = 0.43$).

Natal and breeding dispersal

Parents and their newborn and one-year-old offspring occurred on the same rock outcrop in almost all cases. One juvenile from the one-year-old cohort was assigned with 98% confidence to a female on a rock 356 metres away at site P2. In addition, the newborn offspring of one male occurred on two different rocks at site T1 that were 141 metres apart (both assigned with > 97% confidence).

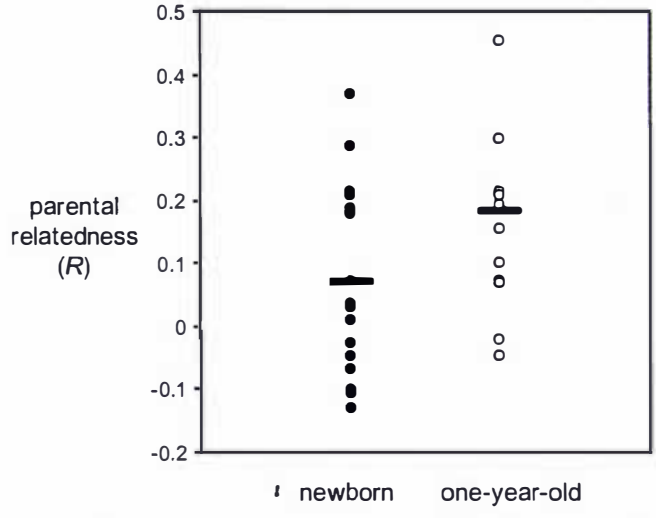

Figure 2. Relatedness of the parents of newborn and one-year-old offspring. Horizontal lines indicate means.

Sex-biased dispersal

There was no difference in relatedness among female and male skinks on outcrops in either pasture (female $R = 0.106 \pm 0.027$ SE vs. male $R = 0.108 \pm 0.053$ SE, $t = 0.25$, $P = 0.61$), or tussock (female $R = 0.054 \pm 0.023$ SE *vs.* male $R = 0.077 \pm 0.074$ SE, $t = 0.14$ $P = 0.15$), or overall (female $R = 0.010 \pm 0.031$ SE vs. male $R = 0.080 \pm 0.043$ SE, $t = 1.22$, $P = 0.25$).

Discussion

The study of inbreeding avoidance has practical relevance for the grand skink because many populations occur in modified pasture-dominated landscapes where the rate of dispersal between rock outcrops is reduced (Chapter 4). Pasture populations are predicted to face an increased chance of inbreeding and its deleterious effects (Frankham, 1995). The magnitude of this effect may depend in part on the mating system of grand skinks and their ability to avoid breeding with kin (Parker & Waite, 1997; Amos & Balmford, 2001; Stow & Sunnucks, 2004), but also their inherent susceptibility to inbreeding effects (Ralls et al., 1988).

Opportunities for inbreeding

Inbreeding avoidance is most likely to evolve when there are opportunities for inbreeding (Ralls et al., 1986; Waldman & McKinnon, 1993). Previous mark-recapture and genetic data indicated that inbreeding is likely in grand skinks because they are sedentary and populations are genetically differentiated at very fine scales ($\leq$ 35 metres, Chapters 3 and 4). Analysis of relatedness confirms this prediction by showing that skinks typically live with many close adult relatives of the opposite sex. Many relationships were equivalent to half sibs (up to 35% of potential mates), but on some rock outcrops adult skinks were also likely to encounter full-sibs (up to 17% of potential mates). This kinship structure emphasises the sedentary nature of grand skinks because sexual maturity occurs in their fourth year (Cree, 1994), providing ample time for dispersal. In addition, close kinship occurs in both natural (tussock grassland) and modified (pasture) landscapes, meaning that risk of inbreeding is characteristic of the species, and is not just a result of habitat modification.

Inbreeding avoidance by mate choice

Theoretical reasons for avoiding inbreeding are clear - matings between relatives increase the proportion of loci that are homozygous in offspring compared to unrelated pairs, and higher homozygosity increases the chance of deleterious recessive alleles being expressed (Falconer, 1989). Reviews by Ralls *et al.* (1986) and Keller & Waller (2002) appeared to confirm that these risks apply to wild populations by showing that many mammals and birds avoid inbreeding. Yet, although adult grand skinks usually live with close relatives, they did not avoid breeding with them. This resulted in a high level of close inbreeding (18.2%), not because inbreeding was systematic, but because there were few unrelated mates to choose from on a rock outcrop. Moreover, that level

of inbreeding observed is much higher than the ≤ 6% value reported from the review of mammals and birds by Ralls *et al.* (1986). The absence of obvious inbreeding avoidance is unlikely to be because lizards lack such discriminatory abilities, as these behaviours have been demonstrated in several lizard species (Léna et al., 2000; Gardner et al., 2001; Stow & Sunnucks, 2004). Therefore, unlike outbreeding species (e.g. Stow & Sunnucks, 2004), grand skinks are not naturally buffered against the potentially deleterious genetic effects of isolation caused by habitat modification. *Oligosoma grande* adds to a growing list of taxa that inbreed despite the theoretical or actual risks (e.g. dwarf mongoose, Keane et al., 1996; song sparrow, Keller & Arcese, 1998; white-toothed shrew, Duarte et al., 2003; glanville fritillary butterfly, Haikola et al., 2004).

Inbreeding avoidance by natal or sex-biased dispersal

Natal (pre-breeding) or sex-biased dispersal are also mechanisms of inbreeding avoidance (Greenwood, 1980). However, parentage and kinship analyses showed that neither natal nor sex-biased dispersal was pronounced in grand skinks, and as indicated by the high proportion of kin present on rock outcrops, neither effectively removed opportunities for close inbreeding. Only one of the 23 one-year-old skinks, whose mother was identified, was captured on a different rock from her. The only other juvenile captured on a different rock from its parent was probably the result of male breeding dispersal because it was captured on the same rock outcrop as its mother, who had been a long-term resident of that rock. This result tallies with mark-recapture data reported in Chapter Three, which showed that only 3.3% of skinks captured just after birth dispersed in their first year of life. I also did not detect a difference in dispersal between males and females based on comparison of male-male and female-female relatedness. However, a more powerful test based on comparison of the mAIc and F_{ST} statistics between males and females (Favre *et al.*, 1997), has previously shown a statistically significant bias towards female dispersal (Chapter 4), and the relatedness data show the same trend. Nevertheless, the female-bias was not pronounced in either of those tests.

Inbreeding avoidance by promiscuity

Promiscuity in lizards has been argued to be a mechanism to avoid inbreeding by enabling females to effect post-copulatory mate choice through sperm competition (Olsson et al., 1996b). Despite their small clutch sizes (average 2.4, Cree, 1994), I detected a high degree of multiple paternity in grand skinks, and also found that males

reproduced with multiple females. Promiscuity is common in reptiles (Olsson & Madsen, 1998), and compares with field observations that male grand skinks interact with multiple females during the breeding season (Murphy, 1994). Females grand skinks store sperm for about five months prior to fertilisation (Cree, 1994), which presumably provides opportunities for sperm competition. Because I did not obtain complete offspring arrays nor identify all males that mated, I could not properly test whether post-copulatory mate choice occurs in grand skinks. However, although promiscuity was common, the proportion of offspring produced by closely related parents was still high, and similar to the proportion of close relatives in the populations studied (Table 1, Figure 2). Therefore, it appears that selection for unrelated mates by either pre- or post-copulatory mate choice is not pronounced in this species.

Costs of inbreeding

Theoretical models show that inbreeding avoidance will evolve when the cost of inbreeding exceeds the cost of avoiding inbreeding (Waser et al., 1986; Lehmann & Perrin, 2003). The cost of inbreeding is often measured in terms of juvenile survival (e.g. Ralls et al., 1988; Olsson et al., 1996a; Keller & Waller, 2002), but I did not detect a cost of inbreeding to skinks surviving in their first year. Instead, I found that skinks that survived their first year were variously inbred, included full sibs (Figures 1 and 2), and were no less inbred than newborn skinks. In fact there was a near significant trend towards one-year-old skinks being more inbred. Had there been strong selection against inbred juveniles, I expected both the mean and variance of parental relatedness of one-year-old skinks to be lower than that for newborn skinks. Although additional components of fitness need to be examined before firm conclusions are drawn (Hedrick & Kalinowski, 2000), the costs of inbreeding appear low in this species. Weak inbreeding depression might be explained by a history of inbreeding that has permitted the purging of deleterious alleles (e.g. Gibbs & Grant, 1989). This scenario is difficult to verify, and empirical evidence of purging in wild populations is scant (Keller & Waller, 2002). However, it is noteworthy that small population size and isolation are conditions conducive to purging, and are also features of grand skink ecology (Whitaker, 1996).

Costs of avoiding inbreeding

The high incidence of close inbreeding in grand skinks may be explained by high costs of avoiding inbreeding, which theory indicates should strongly influence mating

behaviour (Waser et al., 1986). Two potential costs are dispersal, and forgone breeding opportunities (Waser et al., 1986; Keller & Arcese, 1998), and both are potentially important for grand skinks because of their small demic population structure and the patchy nature of rock outcrops. Skinks would reduce opportunities for close inbreeding by dispersing to neighbouring rock outcrops because neighbouring skinks are on average less closely related (Chapter 4). That few skinks take this opportunity implies the cost of dispersal may exceed the cost of inbreeding. Mortality rates of dispersing grand skinks are unknown, and historical levels cannot be measured because feral predators are now abundant in New Zealand. However, dispersal is a significant cause of mortality in many species (Johnson & Gaines, 1990), and given the physical differences between the rock outcrops and the matrix vegetation between them, and the strong affinity skinks have for rock outcrops (e.g. Stanley, 1998), mortality may also be high for dispersing grand skinks.

Despite costly dispersal, skinks might avoid close inbreeding by selecting less related mates from among the candidates on their rock outcrop. Such obligatory outbreeding has been documented in several lizard species that live with close relatives, notably the Australian *Egernia* group skinks (Gardner et al., 2001; Stow & Sunnucks, 2004), yet outbreeding was not observed in grand skinks. This may imply that mate choice is costly. Theoretically, a disadvantage of obligatory outbreeding is the glass effect (Tainaka & Itoh, 1996), where all breeding opportunities are forgone if outbred matings are unavailable. The small and isolated nature of grand skink populations means that opportunities for outbred matings may be limited. For example, the five females on rock P2.2 were related to 40-80% of the males at the level of half-sibs (Figure 1). This may explain the willingness of grand skinks to inbreed, particularly if close inbreeding does not have strongly deleterious effects as the results presented above suggest.

In summary, the results presented here conclusively show that the assumption that animals avoid inbreeding may be unfounded, and further studies are required to establish the true extent of inbreeding in wild populations. In the case of the grand skink, close inbreeding was common in both modified and unmodified landscapes, and had no detectable deleterious effect on offspring survival. Genetic threats (*cf.* Frankham, 1995) need not take high priority for the conservation management of this species in the short term.

Acknowledgements

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Appendix 5.1. Parameters used in the simulation for parentage analysis using CERVUS 2.0.

	1999/00		2000/01		1999/00		2000/01		1999/00		2000/01	
	Tussock 1				Pasture 1				Pasture 2			
	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males
No. of loci	13	13	13	13	14	14	14	14	14	14	14	14
Cycles	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
No. candidate parents	17	17	15	12	16	12	14	8	13	13	13	13
Prop candidate parents sampled	0.530	0.470	0.950	0.600	0.690	0.670	0.950	0.9500	0.600	0.600	0.950	0.800
Prop loci typed	0.973	0.973	0.973	0.973	0.991	0.991	0.991	0.991	0.977	0.977	0.977	0.977
Prop loci mistyped	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020
Number of relatives	1	1	1	1	1	1	1	1	1	1	1	1
Relatedness to candidate	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250

6

Distinguishing historical fragmentation from a recent population decline: shrinking or pre-shrunk skink from New Zealand?*

Abstract

Species that are rare when first described present a practical management problem because it may be unclear whether the taxon is in the final stages of an anthropogenic decline, or is naturally uncommon, and each scenario dictates a distinct approach to management. I analysed mitochondrial and microsatellite DNA data with population genetic and phylogenetic tools to distinguish between these possibilities in a rare lizard from southern New Zealand. Grand skinks, *Oligosoma grande*, are large rock-dwelling lizards that have a fragmented distribution consisting of a western and eastern cluster of populations separated by *ca.* 120 km. This distribution could result from human disturbance, pre-human climatic and vegetation changes, or both. All populations were highly genetically structured (overall F_{ST} 0.171, R_{ST} 0.235), indicating that populations were demographically independent and skinks are unlikely to expand their range without human intervention. In addition, the current fragmented distribution is likely to have both historical and recent anthropogenic elements. Two eastern populations showed evidence of being historically large (high mtDNA genetic diversity), although they are now small, supporting anecdotal data that grand skinks have declined in historical times. However, eastern and western populations were reciprocally monophyletic for mtDNA lineages, suggesting long independent evolutionary histories that predate the arrival of humans in New Zealand. Eastern and western populations fulfil many criteria to be considered as evolutionarily significant units, but such a classification must be balanced against addressing more immediate threats to the species' survival, such as introduced predators.

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"Identifying a decline precedes diagnosing the factors driving it."

Caughley & Gunn, 1996

Introduction

Humans and their commensals have caused the endangerment and extinction of countless species. Although the declines of charismatic or commercially important species and the agents responsible are often well documented (e.g. the northern elephant seal, *Mirounga angustirostris*, Stewart *et al.*, 1994), less obvious species may be rare before they are even described (e.g. the white-bellied frog, *Geocrinia vitellina*, Roberts *et al.*, 1990). Such cases present a practical problem for wildlife managers because it will be unclear whether the taxon is in the final stages of a major decline caused by human activities, or is naturally uncommon, and each scenario dictates a distinct approach to management. For example, if the species has suffered a recent decline, a key to successful conservation will be to identify the causes so that their impacts can be reduced or eliminated. In contrast, if a species is naturally rare, seeking proximate biological causes for rarity such as predation, competition or habitat degradation will be fruitless. Distinguishing declining from naturally rare species is a critical step in the conservation management of species (Caughley, 1994).

Inferring declines

Recent range contractions can be inferred for a few species from subfossil deposits, which may also allow the agents of decline to be identified. For example, the Tuatara, *Sphenodon punctatus*, is now restricted to offshore islands around New Zealand (Towns *et al.*, 2001), yet subfossil bones indicate that it was widespread on the mainland until 1000 years ago (Worthy, 1997). The disappearance of Tuatara from mainland New Zealand coincides with the arrival of Polynesian settlers and the pacific rat *Rattus exulans*. Further experimental work has verified that *R. exulans* has a detrimental effect on Tuatara populations (Cree *et al.*, 1995). Similar patterns exist for many taxa in New Zealand (Whitaker, 1978; Worthy, 1987), the Pacific islands (Pregill, 1989), and Madagascar (Dewar, 1984).

More commonly, fossil records are not available, and the demographic history of an animal species must be inferred by other means. Increasingly, this is by analysis of molecular data (e.g. Avise, 1992; Grant & Bowen, 1998; Goldstein *et al.*, 1999). One

widely employed approach to diagnosing population declines is by comparison of the observed and equilibrium heterozygosities calculated from codominant DNA markers (e.g. Cornuet & Luikart, 1996; Luikart & Cornuet, 1998; Luikart *et al.*, 1998). However, this method is only useful where bottlenecks have been fairly drastic ($N_e \leq 20$ individuals), and have occurred recently (2-4N_e generations; Luikart *et al.*, 1998). Consequently, bottleneck tests are not well suited to identifying ongoing population reduction where a species still numbers in the hundreds or thousands. Yet reversing declines before they get to a critical bottleneck stage is vital to successful conservation (Caughley, 1994).

Range disjunctions may also indicate that a once widespread species has declined, but could equally have arisen from ancient vicariance driven by geological or climatic events (Firestone *et al.*, 1999). One approach to distinguishing these processes is phylogeographical analysis of mitochondrial DNA (mtDNA) sequences (e.g. Carpenter *et al.*, 2001; Williams, 2002). Mitochondrial DNA is well suited to studying demographic processes because its small effective population size ($1/4N_e$) makes it highly responsive to genetic drift. In addition, because mtDNA typically is not subject to recombination, the dynamics of the lineage sorting process and how population demography affects it are well understood (e.g. Avise *et al.*, 1984; Neigel & Avise, 1986; Rogers & Harpending, 1992). In situations where a disjunct range is encountered, a useful qualitative measure of time since isolation is the predicted progression through time from lineage polyphyly via paraphyly to reciprocal monophyly caused by the stochastic sorting of mtDNA lineages (Neigel & Avise, 1986). In some circumstances, the inference can be extended to a more quantitative measure of time since isolation by use of a molecular clock to date lineage divergence (e.g. Waters *et al.*, 2001; Trewick & Wallis, 2001). Mitochondrial DNA data can also provide information about historical effective population sizes because the time to mtDNA lineage coalescence depends on female effective population size (N_{eif}), rate of population growth, and mutation rate (Avise *et al.*, 1984; Avise *et al.*, 1988; Rogers & Harpending, 1992; Kuhner *et al.*, 1998; Crandall *et al.*, 1999). Comparisons of historical effective population size with estimates of current census size enable recent population declines or expansions to be identified (e.g. Ball & Avise, 1992; Roman & Palumbi, 2003).

The case of the grand skink

In this study, I examine a situation where a rare species has a disjunct range that could be explained by either recent anthropogenic landscape changes or more ancient processes associated with pre-Holocene and pre-human climate change. The grand skink, *Oligosoma grande*, is a large rock-dwelling lizard endemic to montane grasslands in southern New Zealand. The holotype specimen was described in 1845 (Hardy, 1977), but few records were made during the following century until systematic distribution surveys were conducted in the 1980s (Whitaker & Loh, 1995). Fewer than 5000 individuals exist, and the species is considered vulnerable to extinction by IUCN criteria (Hilton-Taylor, 2000; Whitaker & Houston, 2002). Grand skinks have a curious disjunct distribution (Figure 1). Most populations occur patchily in a limited region of eastern Otago, but several small populations are also known from western Otago over 120 km away. Aside from a single eastern population at Macraes Flat that consists of several thousand individuals (Patterson, 1992), repeated surveys have revealed that most populations are extremely small and probably consist of tens to hundreds of individuals (G. Loh, NZ Dept. Conservation, pers. comm.).

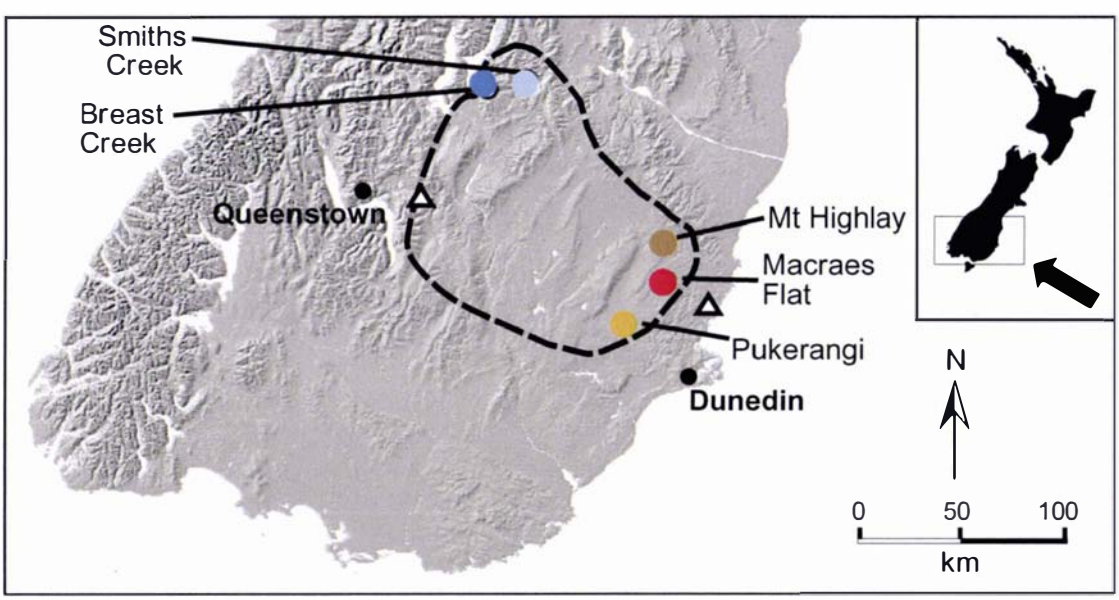

Figure 1. The distribution of grand skinks, *Oligosoma grande,* in southern New Zealand. Coloured circles are sampled populations, and the open triangles are the approximate locations of historical samples. The dashed line indicates the estimated (pre-human) former range (adapted from Whitaker & Houston, 2002), and includes most of the district of Otago.

The current vulnerable status and approach to management of this species is based on the presumption that it was formerly widespread and has suffered a major range decline since human settlement in New Zealand, retracting to its current distribution, in particular, since the introduction of rabbits *Oryctolagus cunicularis*, 200 years ago (Whitaker & Houston, 2002). With little historical documentation, evidence for the decline is largely anecdotal, and mainly based on three points: 1) the occurrence of apparently suitable habitat in the region between the current populations; 2) anecdotal accounts of "large lizards" in central Otago; and 3) two specimens collected outside the current range in 1907 and 1910 (Figure 1; Hardy, 1977; Whitaker & Loh, 1995).

An alternative yet unexplored hypothesis is that grand skink populations experienced vicariance associated with pre-Holocene climatic changes, and that the isolated populations represent ancient refugia, predating human arrival in New Zealand. The arguments for a more ancient cause of the distribution are: 1) in the last 2-5 million years southern New Zealand experienced tectonic mountain building, greatly fluctuating temperatures, extensive glacial activity, and pronounced vegetational changes (Kershaw, 1988; Pillans *et al.*, 1992; Cooper & Millener, 1993; McGlone *et al.*, 1995) that may have affected the distribution of skinks; 2) evidence of morphological differences between individuals from the western and eastern parts of the range, which may be indicative of a long period of separation (Whitaker & Loh, 1995); 3) the presence of a series of mountainous ridges and wide valleys bisecting the region between the eastern and western populations that could limit dispersal, particularly in combination with climatic changes (Figure 1); and 4) several taxa from southern New Zealand exhibit pronounced phylogeographic structure that has been attributed to the dynamic geological history of the region (e.g. geckos, Hitchmough, 1997; invertebrates, Trewick *et al.*, 2000; Trewick & Wallis, 2001; Chinn & Gemmell, 2004; galaxiid fish, Waters *et al.*, 2001). Furthermore, genetic studies of New Zealand lizards have revealed a large number of cryptic species, prompting extensive taxonomic revision (Daugherty *et al.*, 1993; Patterson, 1997; Hitchmough, 1997).

I used sequences from the mtDNA control region and nuclear microsatellite DNA loci to evaluate two related questions: (1) do western and eastern grand skink populations represent ancient refugia that are a legacy of Pleistocene glacial cycles; and (2) is there evidence of recent population declines?

Methods

Sampling

Sixty-five grand skinks were sampled from most known populations throughout the species' range between November 2000 and March 2002 (Figure 1; Table 1). Despite extensive searching, few samples (n = 2) could be obtained from two populations (Mt Highlay and Smiths Creek) where skinks were at extremely low densities (~0.67 skinks sighted day^{-1}). Skinks were captured from rocky outcrops (eastern populations) and small bluffs (western populations) with nylon fishing line nooses. Toe-clips or tail tips (< 2 mg tissue) were taken and stored in liquid nitrogen or 95% ethanol and skinks were released at their point of capture. DNA was isolated by a salting-out procedure (Sunnucks & Hales, 1996).

Table 1. Details of grand skink populations sampled for this study.

Region	Population	Lat/Long	*n*
Eastern	Macraes Flat	45° 26'S, 170° 24'E	43
	Pukerangi	45° 38'S, 170° 09'E	7
	Mt Highlay	45° 17'S, 170° 23'E	2
Western	Breast Creek	44° 36'S, 169° 25'E	11
	Smiths Creek	44° 33'S, 169° 37'E	2

Isolation of mtDNA control region sequences

The hypervariable region I (HVRI) of the mtDNA control region has been widely used to recover intraspecific genealogies because of its relatively rapid rate of sequence evolution (e.g. Wenink *et al.*, 1993). However, control region sequences have only been described from a single (and distantly related) skink species (*Eumeces egregius*; Kumazawa & Nishida, 1999). To identify suitable regions for primer placement in HVRI, I characterised the control region in *O. grande* by long-range PCR anchored to flanking genes, followed by inwards sequencing with primer walking. Initially I sequenced a 306 nt fragment of the mitochondrial cytochrome *b* gene of four skinks

from the Macraes flat population using universal primers (L14841 and H15149; Kocher *et al.*, 1989), and from this fragment I designed the primer cytb-longL (Table 2). In addition, I designed a primer 12s-longH to a conserved region of the 12s rRNA identified by aligning sequences from 25 New Zealand skinks in the genera *Oligosoma* and *Cyclodina* (Hickson *et al.*, 2000). Using these primers I amplified a 3804 nt fragment including partial sequences of 12s rRNA, cytochrome *b*, and entire tRNA-threonine, tRNA-phenylanaline, tRNA-proline and control regions.

Polymerase chain reactions (PCR) were performed in 25 μL volumes consisting of 1 x PCR buffer (Roche expand buffer 3), 2.25 mM $MgCl_2$, 2.0 mM each dNTP, 0.4 μg/μL bovine serum albumin, 2 U *Taq* polymerase (Roche expand), 40 ng genomic DNA, and 0.3 μM of each primer. The PCR profile consisted of one cycle of 94°C for 2 mins, 25 cycles of 94°C for 10 secs, 68°C for 2 min 20 secs, increasing the extension by 20 secs per cycle after the first 10 cycles, and a final extension at 68°C for 7 mins. PCR was carried out on an iCycler thermal cycler (Biorad). Using the long range PCR product as a template I used 8 sequencing primers to walk inwards from the 5' and 3' ends to obtain the entire sequence (Table 2; sequence deposited in Genbank as accession number AY737396).

Two areas of heteroplasmy caused by repeat regions within the control region were observed between primers tRNAp-L and CR-1H. To obtain clean sequences for this region I amplified products with these primers, cloned them into JM109 High efficiency cells using a pGEMt easy kit (Promega), and sequenced 6 recombinant clones. I designed primers OgHVR1-L and OgHVR1-H to amplify a 607 nt fragment of the HVRI. Twenty-five microlitre PCR reactions consisted of 1 x PCR buffer (Roche), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4μg/μL bovine serum albumin, 1 M Betaine, 0.5 U *Taq* polymerase (Roche), 50 ng genomic DNA, and 1.0 mM each primer. The PCR profile consisted of one cycle of 94°C for 2 mins, 35 cycles of 94°C for 30 secs, 58.5°C for 20 secs, 72°C for 30 secs, and a final extension at 72°C for 5 mins. Sequencing was carried out using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit.

MtDNA analysis

Sequences were aligned using CLUSTALX (Thompson *et al.*, 1997). Seventeen unique haplotypes were identified from 530 nt of sequence that could be resolved from all

individuals (Table 3). Nucleotide and haplotype diversity were estimated using REAP 4.0 (McElroy *et al.*, 1991) according to equations 10.5 and 8.4 respectively in Nei (1987). I used the program ARLEQUIN 2.000 (Schneider *et al.*, 2000) to estimate pairwise F_{ST} values between populations from haplotype frequencies.

Table 2. Position of primers used in this study for PCR amplification and sequencing of the mtDNA control region and flanking sequences.

Primer Name	Sequence 5'-3'	Primer 5' position*
cytb-longL	ATCAGCATTCTCATCTCTCGCCCACATC	110
12s-longH	ATAGGCTGAGTTTCAAAAAGTGGTGAGG	N/A
cytb-2L	GCCTACGCAATCCTACGATCCATTCC	759
tRNAp-L	GCTAACCCCTCGTCACTAACTCC	1154
tRNAf-H	AAGTCCCATCTCAGCATCTTCAG	2957
CR-1H	CACTGAAAGGTGGCTGAGGTC	2261
OgHVRI-L	CCTCCATTCCCACTCCCTTTCC	1425
OgHVRI-H	GTTGAGAAACCAGTAGACTTCC	2076

*Numbers refer to position of 5' end of primer relative to the total fragment (5'-3' on the light strand; Genbank accession number AY737396).

Prior to phylogenetic analysis I used the computer program MODELTEST 3.06 (Posada & Crandall, 1998) to determine the appropriate model of sequence evolution for the data. I used unweighted maximum parsimony (MP), and maximum likelihood (ML) analysis to infer phylogenetic relationships among haplotypes using the program PAUP* 4.01 (Swofford, 1998). For the ML analysis I conducted an initial heuristic search using the tree bisection and reconnection (TBR) option with ten random repeats of sequence addition, then reoptimised the model parameters on this tree before running a final heuristic search. A heuristic search was also conducted for the maximum parsimony criteria using TBR with ten random additions of taxa. Support for the branching pattern in the ML analysis was established using 100 nonparametric bootstrap replicates, and the MP analysis with 1000 replicates. PAUP* was also used to calculate a pairwise distance matrix between all haplotypes using the HKY85 + Γ model of substitution. I tested for clock-like sequence evolution using a likelihood difference test after removing the outgroup sequence (Huelsenbeck & Crandall, 1997).

Effective population size estimates

I used a maximum likelihood approach implemented in the computer program FLUCTUATE 1.4 (Kuhner *et al.*, 1998) to estimate the genetic diversity parameter θ in the Macraes Flat and Pukerangi populations, for which estimates of census size were available. This method makes use of the information about a population's history contained in genealogical data and allows for genealogical uncertainty by sampling over a range of genealogies weighted by their likelihoods to estimate θ. θ is a product of the female inbreeding effective population size ($2N_{eif}$) and the substitution rate per generation (μ), and thus if substitution rate is known, θ can be used to estimate the effective population size (see Vilá *et al.*, 1999; Roman & Palumbi, 2003). Substitution rates for the mtDNA control region have not been characterised in lizards. I use a relatively fast estimate of 2.85×10^{-8} s/s/yr (=2.85% pmy) taken from cytochrome *b* sequence of a lacertid lizard ("fast clock", Paulo *et al.*, 2001). This rate is the most rapidly evolving molecular clock that has been published for a lizard, and therefore represents a conservative lower bound for the rate of evolution in *O. grande*. In addition, because it is generally accepted that the mtDNA control region evolves 3-5 times as rapidly as protein coding genes such as cytochrome *b* (Aquadro & Greenberg, 1983; but see Ruokonen & Kvist, 2002), I also use 1.0×10^{-7}, to cover the likely range of substitution rates in skinks. Generation time was estimated from the average age of female skinks giving birth, which was 6.4 years (O. Berry unpubl. data). FLUCTUATE assumes no selection, recombination or migration and I also assumed a constant population size. The microsatellite data indicated that gene flow between populations was negligible. However, for population Macraes Flat there was evidence of historical gene flow, which would inflate θ estimates (see results). Therefore I calculated two values of θ – one that included lineages that might have originated from outside Macraes Flat, and another without these lineages. I used estimates for Ti/Tv ratio and base frequencies as estimated from a run of MODELTEST as starting values for the analysis.

Hypothesis testing

I used parametric bootstrapping (Hillis *et al.*, 1996) to test the null hypothesis that eastern and western populations were reciprocally monophyletic for mtDNA lineages. A model tree was selected using a maximum parsimony search with the dataset constrained to conform to the reciprocal monophyly hypothesis. I used the difference between the parsimony score of the best constrained MP tree and the best unconstrained

MP tree as the test value. I then generated 100 simulated sequence datasets using maximum likelihood estimates of model parameters and branch lengths from the best constrained MP tree using the computer program SEQ-GEN (Rambaut & Grassley, 1997), and conducted two heuristic searches on each dataset. The first was to find the best tree overall, and the second to find the best tree compatible with the constraint. The differences in parsimony scores for each dataset were used to construct a null distribution of these differences. The significance of the test was assessed by direct comparison of the test value for the actual data with the null distribution (Hillis *et al.*, 1996; Goldman *et al.*, 2000).

Microsatellite Analyses

All skinks were genotyped with 12 microsatellite loci designed for *O. grande* (Chapter 2). Skinks from the western populations did not amplify at two loci (Oligr11 and Oligr15) and these loci were removed from the analysis. I calculated mean and pairwise F_{ST} (Weir & Cockerham, 1984) between populations with FSTAT 2.9.3 (Goudet, 1995), and also calculated values of weighted R_{st} (Rousset, 1996), which accounts for stepwise mutation in microsatellite DNA. In addition, I calculated the shared allele distance between all pairs of individuals (D_{as}), and constructed a Neighbour-Joining (NJ) tree using the program POPULATIONS 1.2.28 (Langella, 2000). I also constructed a NJ tree based on the average D_{as} between populations. Support for the nodes of this tree was established by bootstrapping over loci and over individuals.

Results

I characterised a 3804 nucleotide sequence of the grand skink mtDNA genome. This consisted of the majority of the cytochrome *b* gene, tRNA-phenylalanine, tRNA-threonine, the control region, tRNA-proline, and 383 nt of the 12s ribosomal RNA gene.

Sequence heteroplasmy in the mtDNA control region

The control region contained two heteroplasmic regions. The first, at the 5' end of HVRI, consisted of 50 nt repeat units. In two clones sequenced from a single skink one had a single motif and the other had four identical repeats. In addition, in two skinks sequenced for this region, two different repeat motifs were identified. The repeats differed by an A↔T and a C↔T substitution. Heteroplasmy was also observed in a poly-A string near the central conserved domain. In six clones sequenced from a single individual A_{10}, A_{11}, A_{12} and A_{13} haplotypes were recorded.

Characteristics of the mtDNA data

530 nt of control region sequence could be resolved from all individuals and I detected 47 polymorphic sites (Table 2). Thirty-six sites were parsimony informative, and eleven were autapomorphies. The most likely model of nucleotide substitution as identified by MODELTEST was HKY85 + Γ (Appendix 6.1). The estimated nucleotide frequencies in the dataset according to this model were A: 0.298, C: 0.093, G: 0.259, T: 0.350, the gamma shape parameter was 0.1737, and the transition to transversion ratio was 3.5689:1. The haplotype sequences have been deposited in Genbank as accession numbers AY700064-AY700080.

Table 2. Variable sites among *Oligosoma grande* control region mtDNA haplotypes. Haplotype sequences are deposited in genbank as accessions AY700064-AY700080).

	Position																																															
Haplotype	094	109	112	141	147	155	156	162	189	190	191	192	215	219	227	242	260	261	265	270	271	273	274	296	299	343	351	354	355	379	387	413	418	419	420	438	448	454	456	467	469	470	477	478	492	494	514	n	Pop
H-Og1	A	T	T	A	G	G	A	T	A	G	A	G	T	A	A	T	T	T	A	T	G	A	A	T	G	G	G	G	C	A	A	G	T	A	G	G	A	A	A	A	A	C	C	A	T	T	T	14	MF
H-Og2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	7	MF
H-Og3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	6	MF
H-Og4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	6	MF
H-Og5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	2	MF
H-Og6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	2	MF
H-Og7	.	.	.	.	.	A	G	C	.	T	.	.	.	G	.	C	.	.	T	.	A	G	.	.	.	C	.	A	T	.	.	A	A	G	.	A	.	G	.	G	G	.	T	.	G	.	.	1	MF
H-Og8	.	.	.	.	A	A	.	.	.	T	.	.	.	G	G	C	.	.	T	.	A	G	.	.	.	C	.	A	T	.	.	A	.	.	.	A	.	G	.	G	G	T	T	.	G	.	.	1	MF
H-Og9	.	.	C	G	.	A	.	.	G	T	.	A	.	.	.	.	C	.	T	A	A	.	.	G	.	.	A	A	.	G	.	A	.	.	A	.	G	G	.	.	.	.	T	.	G	.	C	2	MF
H-Og10	.	.	.	.	.	A	.	.	.	.	.	A	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	3	PK
H-Og11	.	.	.	.	.	A	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	PK
H-Og12	.	.	.	.	.	A	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	1	PK
H-Og13	.	.	.	.	.	A	.	.	.	.	.	A	.	G	.	.	.	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	1	PK
H-Og14	.	.	C	G	.	.	.	.	G	T	.	A	.	.	.	.	C	.	T	A	A	.	.	G	.	.	A	A	.	G	.	A	.	.	A	.	G	G	.	.	.	.	T	.	G	.	C	2	MH
H-Og15	T	A	.	.	.	.	.	.	.	T	G	A	.	.	.	.	.	.	T	.	A	.	.	.	T	C	.	A	.	G	.	A	.	.	.	.	.	G	.	G	G	T	T	.	G	.	.	8	BC
H-Og16	T	A	.	.	.	.	.	.	.	T	G	A	.	.	.	.	.	.	T	.	A	.	.	.	T	C	.	A	.	G	.	A	.	.	.	.	.	G	.	G	G	T	T	G	G	.	.	1	BC
H-Og17	T	.	.	.	.	.	.	.	G	T	.	A	.	.	.	.	.	.	T	.	A	.	.	.	.	C	.	A	.	G	G	A	.	.	.	.	.	G	.	G	G	T	T	.	G	.	.	2	SC

MtDNA phylogenetic analyses

No populations shared haplotypes (Table 2). Both eastern populations Pukerangi and Macraes Flat had high haplotypic and nucleotide diversity relative to the western population at Breast Creek (Table 3). The Macraes Flat population contained three groups of highly divergent haplotypes, with corrected HKY85 distances between haplotypes ranging between 0.2 and 6.4% (average 3.1% ± 0.4 SE, Appendix 6.2). Trees produced by maximum likelihood and maximum parsimony criteria had very similar topologies, resolving the same four well supported clades (Figure 2), but neither analysis could resolve whether eastern and western populations were reciprocally monophyletic. However, the most parsimonious tree constrained to the reciprocal monophyly hypothesis only required an additional three steps more than the observed tree, and parametric bootstrapping analyses indicated that the null hypothesis of reciprocal monophyly of eastern and western populations could not be rejected (P = 0.53). The likelihood difference test showed no significant difference between the log-likelihood of phylogenetic trees with and without a molecular clock enforced (2Δlog-likelihood = 23.54, critical $\chi^2_{15} = 25.00$, $P > 0.05$).

θ and historical effective population sizes

Values of the genetic diversity estimator θ for populations Macraes Flat and Pukerangi were high (Table 4), and translated into estimates of effective female population size that were greater than census sizes in most cases, regardless of the assumed substitution rate or whether putative historical migrant haplotypes were included.

(a) maximum parsimony

(b) maximum likelihood

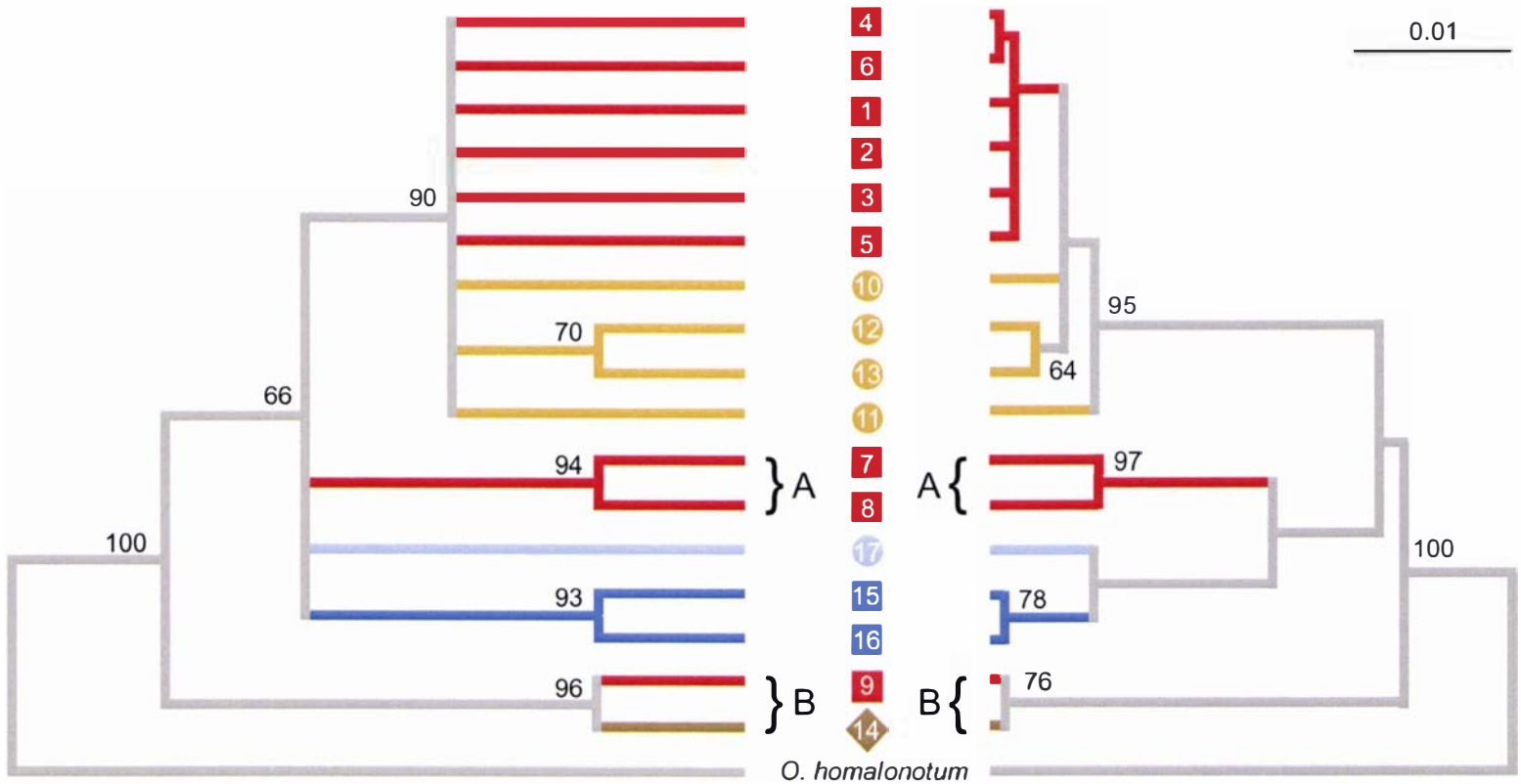

Figure 2. Phylogenetic trees based on control region mtDNA sequence from *Oligosoma grande*. (a) Maximum parsimony analysis showing strict consensus of 3750 most parsimonious trees with 198 steps. (b) Maximum likelihood analysis under a HKY85 + Γ model of sequence evolution (majority rule bootstrap consensus). Numbers above nodes are non-parametric bootstrap values. Symbols identify populations: red square Macraes Flat, orange circle Pukerangi, brown diamond Mt Highlay, dark blue square Breast Creek, light blue circle Smiths Creek. The lineages labelled A and B are referred to in the text, and the numbers inside the symbols refer to haplotypes in Table 3.

Table 3. Descriptive statistics for microsatellite and mtDNA data

			microsatellite				mtDNA			
Population		*n*	H_{exp}	H_{obs}	AR	*A*	hap	*h*	*Π*	Dist[1]±SE
eastern	MF	42	0.82±0.04	0.78±0.11	3.14±0.14	11.5±1.0	9	0.82±0.04	0.02541	3.1±0.4
	PK	7	0.90±0.03	0.64±0.18	3.37±0.12	7.7±0.7	4	0.80±0.17	0.00693	0.8±0.1
	MH	2	0.60±0.09	0.60±0.32	2.30±0.21	2.3±0.2	1	0	-	0
western	BC	11	0.70±0.04	0.50±0.16	2.62±0.13	5.2±0.4	2	0.22±0.17	0.00043	0.2±0.2
	SC	2	0.78±0.08	0.75±0.26	2.90±0.28	2.9±0.3	1	0	-	0
Total		64	0.78±0.02	0.65±0.03	3.4±0.09	20.8±1.9	17	0.37±0.03		3.3±0.2

AR = allelic richness (based on a maximum of 2 diploid individuals), H_{exp} = gene diversity, H_{obs} = observed heterozygosity, *A* = average number of alleles, hap = number of haplotypes recorded, *h* = haplotype diversity, *Π* = nucleotide diversity, [1]Dist = average HKY85 + Γ distance between haplotypes within population.

Table 4. Estimates of female effective population size (N_{eif}) at Macraes Flat and Pukerangi populations derived from estimates of θ. Partial haplotypes refers to calculations based on haplotypes H-Og1-6 only, and all haplotypes refers to calculations based on all haplotypes recorded from Macraes Flat (see text for explanation).

Population/clades included	Substitution rate/gen (μ)	Genetic diversity (θ)	Effective female population size (N_{eif})	95% confidence intervals of N_{eif}	Census size
Macraes Flat					1800[1]
partial haplotypes	1.84×10^{-7}	0.0034	9,170	3,170-13,351	
	6.44×10^{-7}		2,613	903-3,805	
Macraes Flat					
all haplotypes	1.84×10^{-7}	0.0196	53,304	34,624-82,134	
	6.44×10^{-7}		15,192	9,868-23,408	
Pukerangi					100s[2]
	1.84×10^{-7}	0.0079	21,584	7,143-76,146	
	6.44×10^{-7}		6,151	2,036-21,702	

[1]Patterson, 1992, [2]Graeme Loh, NZ Department of Conservation pers. comm.

Microsatellite DNA data

All populations exhibited high levels of microsatellite DNA variation (Table 3), although the eastern populations Mt Highlay and Pukerangi were genetically more variable than the only well-sampled western population at Breast Creek based on all measures of diversity. Paired t-tests based on allelic richness, which accounts for differences in sample size (Petit *et al.*, 1998), showed that both Macraes Flat and Pukerangi were significantly more variable than Breast Creek (Macraes Flat vs. Breast Creek $t_{9,\ 0.05} = 4.83$, $p < 0.001$; Pukerangi vs. Breast Creek $t_{9,0.05} = 2.26$, $p < 0.001$). Sample sizes for the Mt Highlay and Smiths Creek populations were too small for useful comparisons to be made.

The overall level of genetic subdivision among populations as measured by F_{ST} was 0.171 (± 0.022 SE), and by weighted R_{st} was 0.235 (± 0.082 SE). All populations were highly genetically divergent, although the small samples from Smiths Creek and Mt Highlay mean that the results for these populations are provisional (Table 5). In addition, clustering analysis showed that all individuals grouped according to their population of origin (Figure 3). Clustering analysis of populations showed that the two western populations (Breast Creek and Smiths Creek) and two of the eastern populations (Macraes Flat and Pukerangi) grouped separately from one another with relatively high bootstrap support, but the position of the Mt Highlay population relative to these clades was not well resolved (Figure 4).

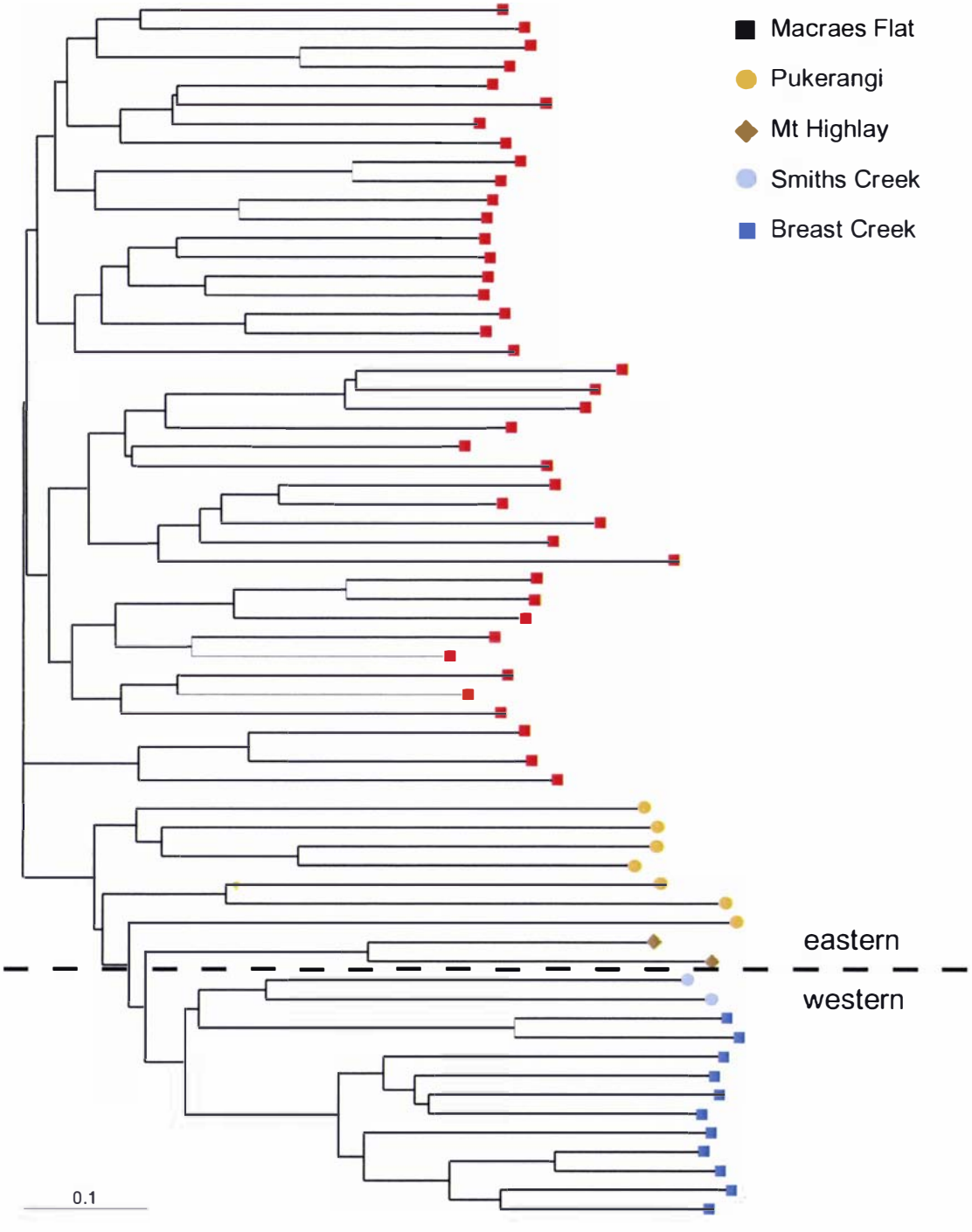

Figure 3. Neighbour joining tree based on the shared allele distance between individuals (D_{as}) calculated from microsatellite DNA data. Symbols refer to sampling populations and are the same as those used in Figure 2.

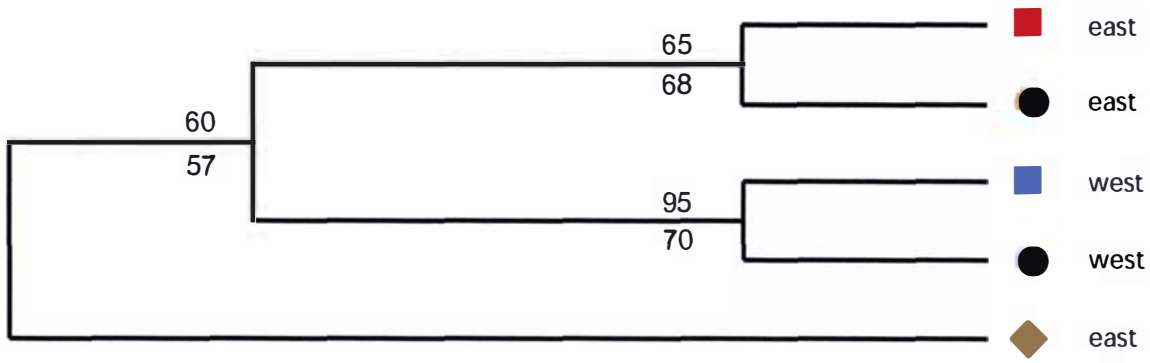

Figure 4. Neighbour joining tree based on the average shared allele distance between populations (D_{as}) calculated from microsatellite DNA data. Numbers at nodes refer to bootstrap support. Values above and below nodes were derived by bootstrapping over individuals and loci respectively. Symbols indicate populations and are the same as those used in Figure 2.

Table 5. Genetic differentiation between grand skink populations. Proportion of shared microsatellite alleles above diagonal and F_{ST} below diagonal (microsatellite / mtDNA).

	Macraes Flat	Pukerangi	Mt Highlay	Breast Creek	Smiths Creek
Macraes Flat		0.42	0.20	0.12	0.22
Pukerangi	0.09 / 0.18		0.29	0.27	0.33
Mt Highlay	0.20 / 0.32	0.13 / 0.40		0.35	0.16
Breast Creek	0.21 / 0.38	0.17 / 0.53	0.22 / 0.81		0.43
Smiths Creek	0.16 / 0.32	0.08 / 0.40	0.29 / 1.00	0.16 / 0.81	

Discussion

Are grand skink populations natural relics or recent fragments? Distinguishing which of these scenarios is more likely will have practical and potentially financial importance for the management strategy adopted for this species. For example, captive breeding and translocation have been considered as possible management tools (Whitaker & Houston, 2002). If the current populations represent lineages that persisted in ancient refugia, their evolutionary distinctiveness may demand that animals from different populations are not interbred or that existing historical relationships among populations are taken into account if translocations are made or captive colonies assembled (Dunham & Minckley, 1998; Moritz, 1999). Alternatively, recently formed and small isolates that are vulnerable to stochastic demographic or genetic processes may be supplemented by translocations from less vulnerable populations without risk of disturbing adaptive gene complexes (e.g. the Florida panther, *Felis concolor coryi*; Hedrick, 1995).

Eastern populations – genetically diverse and a recent decline

Despite their small size (*ca.* 100s – 1000s of individuals), the eastern populations at Macraes Flat and Pukerangi were characterised by high haplotypic, nucleotide and θ diversity. In part this is due to the presence of deeply divergent lineages in the Macraes Flat population, which most likely result from historical gene flow (see below). However, even when these lineages are excluded from analysis, both the Macraes Flat and Pukerangi populations are currently too small to have retained the levels of mtDNA genetic diversity recorded (Table 4). Importantly, this suggests that the current population sizes are a fraction of historical effective population size (N_{eif}). Furthermore, because adult sex ratios are approximately 1:1 (Whitaker, 1996), and effective population sizes are on average only 10% of adult census sizes (Frankham, 1995a), the historical census sizes would have been even greater than the N_{eif} estimates in Table Four. While the estimates of long-term effective female population size make several simplifying assumptions and rely on a single locus, the magnitude of the difference between observed and expected population sizes based on coalescent theory is large enough that the qualitative result appears robust. It is also congruent with anecdotal evidence for a widespread decline of grand skinks (Whitaker & Loh, 1995). Studies that have employed similar approaches to understand demographic histories of populations have usually shown the opposite pattern to that observed here – effective female population sizes much smaller than observed census sizes (e.g. Avise *et al.*,

1988; Ball & Avise, 1992; but see Roman & Palumbi, 2003), but the species examined (e.g. American eel, redwing blackbird) were numerous, geographically widespread and not known to be in decline.

The finding that at least two grand skink populations have declined recently is not unexpected, given that almost half of New Zealand's lizard species (26 of 62) occur as small populations or are restricted to offshore islands (Towns *et al.*, 2001). Nevertheless, before this study, no direct evidence had been gathered that supported the theory put forward by Whitaker and Loh (1995) that grand skinks have declined markedly since human settlement in New Zealand. The results presented here reinforce the need to address the causes of grand skink endangerment, which include predation by feral animals, destruction of habitat and changes to landscape structure (Whitaker & Loh, 1995; Whitaker, 1996; Chapter 4). More generally, the results provide insight into the New Zealand biota prior to human settlement, where in the absence of terrestrial mammals, birds and reptiles may have existed at abundances that are now almost inconceivable. The results also illustrate how genetic evidence can be used to detect a decline even before it reaches a critical bottleneck stage, which is the most relevant time to take management action (Caughley, 1994). In this case tests based on departure from expected equilibrium heterozygosities in the microsatellite data (Cornuet & Luikart, 1996; Luikart *et al.*, 1998) did not show any evidence for a population bottleneck in any grand skink populations (analyses not presented).

Population structure

Grand skink populations were highly genetically structured. By implication, inter-population dispersal must be negligible or zero. This is consistent with results from mark-recapture and fine-scale genetic studies, which show that grand skinks have limited dispersal capabilities on scales of several hundred metres (Houghton, 2000; Chapter 3 and 4). The significance of extremely limited dispersal is twofold. First, in combination with the small size of most populations, almost all face grave risk of extinction by stochastic processes (Lande, 1988), let alone the risks posed by abundant feral predators (Middlemiss, 1995). Second, their ability to recolonise vacant habitat will be very limited, and although suitable rocky habitat is abundant in southern New Zealand (Whitaker & Loh, 1995), they are unlikely to expand their range rapidly without human intervention. This isolation is likely to be exacerbated by the increasing modification of the native vegetation in the region (Chapter 3). Each population clearly

represents a unit of management because their short-term viability is determined entirely by the internal rates of birth and death (Moritz, 1994b).

Eastern populations - deep lineages, ancient vicariance and ancient gene flow

Three highly distinct and well-supported mtDNA lineages were observed in skinks from Macraes Flat. This pattern is uncommon, and is usually interpreted as admixture of historically isolated populations (Grant & Bowen, 1998; Avise, 2000). There has been little characterisation of a molecular clock for lizards, but to provide a rough perspective on the time frame involved in the vicariance I use a relatively rapidly evolving molecular clock estimated from the cytochrome *b* gene of lacertid lizards ("fast clock", Paulo *et al.*, 2001), which evolves at 2.85% per million years. Based on this rate, the divergent mtDNA lineages at Macraes Flat, which differ from one another by a minimum and maximum distance of 4.6 and 6.4% corrected sequence divergence respectively, coalesce between 1.6 and 2.24 million years ago. Significantly, this straddles the Pliocene-Pleistocene boundary (1.8 mya Fleming, 1979), which marks the end of relatively stable warm climates that had persisted since the Miocene, and the beginning of more extreme conditions associated with glacial cycles (Fleming, 1979; Kershaw, 1988; Pillans *et al.*, 1992). Although the cytochrome *b* gene is generally thought to evolve more slowly than the control region (Aquadro & Greenberg, 1983), it does not always (Ruokonen & Kvist, 2002). The dates suggested here must therefore be viewed as provisional until further details of rates of molecular evolution in lizards become available.

Fossil evidence has shown that the Pleistocene glacial conditions profoundly affected the distribution of the New Zealand biota (Suggate *et al.*, 1978; Fleming, 1979; McGlone, 1988), and the molecular data presented here provide an independent demonstration of these impacts. These results closely parallel data from the alpine weta, *Hemideina maori*, (Orthoptera) from the Rock and Pillar Range, which is immediately to the east of Macraes Flat (King *et al.*, 2003). In that study, two deeply divergent mtDNA clades with corrected mtDNA COII distance of *ca.* 4.5% are sympatric and are different colour morphs. This indicates that a common climatic process at the Pliocene-Pleistocene boundary may have caused both alpine invertebrates and sub-alpine skinks to retract to multiple refugia. Investigation of the phylogeographic structure of other co-occurring but more common species may reveal further details on the nature of this significant biogeographic processes.

The mtDNA data also offer a perspective on the nature and timing of the expansion from these refugia. The presence of three divergent mtDNA lineages at Macraes Flat implies that at least two separate gene flow events occurred, and from different source populations. In addition, the two lineages (labelled A and B in Figure 2) differ in the level of intra-lineage divergence between haplotypes - haplotypes in lineage A differ by 1.5% corrected sequence difference (see Appendix 6.2), whilst haplotypes in lineage B differ by only a single substitution (0.2%), implying that the geneflow occurred at different times. Since geneflow must have occurred prior to the divergence of haplotypes (*cf.* Bowen & Avise, 1990), the deep divergence in lineage A places it at *ca.* 525,000 years bp (significantly, a similar level of intra-lineage divergence was presented in King *et al*'s (2003) study of weta). In contrast, the high similarity of lineage B haplotypes, which were recorded from both Macraes Flat and Mt Highlay, indicates that gene flow occurred from Mt Highlay to Macraes Flat much more recently, perhaps even during the current interglacial. Additional sampling of the Mt Highlay population would provide greater precision on the timing of this process.

Population history: western populations – post glacial founding

In contrast to the eastern populations, the western population at Breast Creek had extremely low levels of haplotype and nucleotide diversity as well as lower microsatellite diversity. Such a pattern is typical of a historical population bottleneck or founding event (Grant & Bowen, 1998). Because of their close proximity to alpine regions and major glacial valleys such as Lake Hawea, which were periodically covered by extensive ice sheets and tundra during the Pleistocene (Fleming, 1979; Pillans *et al.*, 1992) it is likely that the western populations were founded following the last glacial maximum 14,000 years ago, although because the mtDNA lineage is distinct from all others, the refugium was not shared by any of the eastern populations studied here. Further samples from the Smith Creek population would clarify its relationship to the Breast Creek population.

Pre-human distribution

Whitaker and Loh (1995) suggested that prior to European settlement in New Zealand approximately 200 years ago, grand skinks had a more or less continuous distribution throughout the central Otago region, and have since contracted to approximately 8 % of their former range. The molecular data allow two observations on the pre-human

distribution of grand skinks to be made. First, prior to the Holocene (*ca.* 10,000 ybp) grand skinks had a dynamic distribution that probably changed with the twenty or so Pleistocene glacial cycles that occurred (Suggate *et al.*, 1978). Second, there is evidence that as recently as the Holocene, skink populations that now appear isolated (e.g. Macraes Flat and Mt Highlay) probably experienced gene flow – indicating a previously more continuous distribution. However, the high level of genetic structuring among the remaining populations makes it more likely that the distribution throughout Otago was naturally discontinuous, although the species could still have been widespread. An alternative interpretation that I cannot exclude is that the distribution was continuous, but a very strong isolation by distance effect existed because of the low dispersal capabilities of grand skinks.

The significance of evolutionary history to grand skink management

Do the western and eastern grand skink populations have long-independent evolutionary histories that predate human arrival in New Zealand? The answer is almost certainly yes. Both the mtDNA and microsatellite data indicate that they are strongly genetically differentiated, and they are indistinguishable from being reciprocally monophyletic for mtDNA lineages. In addition, preliminary evidence suggests that western and eastern skinks are subtly morphologically different (size and colour differences; Whitaker & Loh 1995; O. Berry, pers. obs.). Overall, western and eastern populations probably fulfil most criteria to be considered evolutionarily significant units (Waples, 1991; Vogler & Desalle, 1994; Moritz, 1994a; Crandall *et al.*, 2000; Fraser & Bernatchez, 2001). They are also more genetically divergent than almost all eight species of the *Galaxias vulgaris* species complex that are endemic to southern New Zealand rivers (Waters & Wallis, 2001). Yet is this sufficient for managers to give consideration to their evolutionary heritage? I believe that this case highlights the potential for conflict between short-term demographic versus long-term evolutionary goals in conservation.

Like all native New Zealand terrestrial vertebrates, grand skinks face the well-documented pressing threats of predation by invasive mammals and small population size (Towns, 1985; Towns *et al.*, 2001; Whitaker & Houston, 2002). All but one of the populations studied here were small, and although precise population estimates are not known, all must face serious threat of extinction in the short term (Whitaker & Houston, 2002). A problem confronting managers is how to evaluate the relative importance of these known threats alongside less tangible long-term conservation goals such as

ensuring future evolution (Crandall *et al.*, 2000) and avoiding outbreeding depression (Frankham, 1995b). Both the short and long term goals are stated in policy that guides implementation of the New Zealand Conservation Act (1987) (Anonymous, 2003). In practice, managers will have to be pragmatic in implementing the Conservation Act because ascribed evolutionary significance will have little conservation relevance for a species with more immediate threats. This situation parallels one in the management of New Zealand endemic frogs (*Leiopelma spp*. Holyoake *et al.*, 2001), and probably many other taxa.

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Appendix 6.1. MODELTEST 3.06 hierarchical analysis of substitution models for the *Oligosoma grande* mtDNA data.

Null model (H_0)	H_0 vs. H_1	$-\ln L_0$	$-\ln L_1$	d.f	*p*
equal base frequencies	JC69[1] vs. F81[2]	1616.17	1556.60	3	<0.000001
equal ti/tv rates	F81 vs. HKY85[3]	1556.60	1531.72	1	<0.000001
equal ti rates	HKY85 vs. TrN[4]	1531.72	1530.78	1	0.170292
equal tv rates	HKY85 vs. K81[5]	1531.72	1531.34	1	0.381043
equal rates among sites	HKY85 vs. HKY85 + Γ	1531.72	1502.40	1	<0.000001
no invariable sites	HKY85 + Γ vs. HKY85 + Γ + I	1502.40	1500.36	1	0.021737

[1]Jukes & Cantor, 1969, [2]Felsenstein, 1981, [3]Hasagawa et al., 1985, [4]Tamura & Nei, 1993 ,[5] Kimura, 1981.

Appendix 6.2. HKY + Γ genetic distances (%) between seventeen control region mtDNA haplotypes observed in *Oligosoma grande*. Haplotype labels are the same as those listed in Table 2.

population	haplotype	H-Og1	H-Og2	H-Og3	H-Og4	H-Og5	H-Og6	H-Og7	H-Og8	H-Og9	H-Og10	H-Og11	H-Og12	H-Og13	H-Og14	H-Og15	H-Og16
MF	H-Og1																
MF	H-Og2	0.19															
MF	H-Og3	0.19	0.39														
MF	H-Og4	0.39	0.60	0.19													
MF	H-Og5	0.19	0.39	0.39	0.60												
MF	H-Og6	0.19	0.39	0.39	0.19	0.39											
MF	H-Og7	5.24	5.59	4.91	5.24	4.91	5.58										
MF	H-Og8	4.99	5.33	4.66	4.99	4.66	5.33	1.47									
MF	H-Og9	5.24	4.91	4.91	5.24	4.91	5.58	6.40	6.13								
PK	H-Og10	1.24	1.47	1.02	1.24	1.02	1.47	4.53	4.28	4.53							
PK	H-Og11	1.24	1.24	1.47	1.71	1.47	1.47	5.86	5.60	5.51	1.23						
PK	H-Og12	0.81	0.60	1.03	1.26	1.03	1.03	5.24	4.99	4.59	0.80	0.59					
PK	H-Og13	1.03	1.26	1.26	1.49	1.26	1.26	5.59	5.33	5.58	1.02	0.59	0.60				
MH	H-Og14	4.91	4.58	4.58	4.90	4.58	5.24	6.78	6.51	0.19	4.85	5.86	4.91	5.94			
BC	H-Og15	4.19	4.49	3.90	4.19	3.90	4.49	4.03	3.20	4.53	4.15	5.39	4.80	5.12	4.23		
BC	H-Og16	4.49	4.80	4.19	4.49	4.19	4.80	4.33	3.49	4.85	4.45	5.73	5.12	5.45	4.53	0.19	
SC	H-Og17	3.98	4.28	3.68	3.98	3.68	4.28	3.83	3.02	3.72	3.93	5.18	4.59	4.92	3.44	1.01	1.23

Overview

Caughley (1994), in his critique of conservation science, recognised that ecological research can make two key contributions to species conservation: first, recognition of species in decline, and second, diagnosis of the mechanisms driving a decline. Targeted management actions can then eliminate the causes of endangerment. In this context, here I draw together the key findings of this thesis and comment on their implications for the management of the grand skink, *Oligosoma grande*.

Has Oligosoma grande declined?

Although it is self-evident that only species in decline and at risk of extinction require conservation management, in practice, recognising such species is not always straightforward. Grand skinks are uncommon and have a disjunct distribution. The standard interpretation of this pattern has been that the species recently declined in response to human settlement (Whitaker & Loh, 1995). Indeed, there are many precedents for recent catastrophic declines of native species in New Zealand (Atkinson & Cameron, 1993), but there was previously little direct evidence to support the claim for *O. grande*.

I analysed data from microsatellite and mitochondrial DNA markers (developed in Chapters 2 and 6) to understand the contributions of historical and contemporary processes to *O. grande*'s rarity and distribution. I presented evidence that some populations have almost certainly contracted since human settlement because they retain high mtDNA diversity (indicative of large populations) but are now small. This result affirms the vulnerable status of *O. grande,* and reinforces the need to diagnose and eliminate the causes of endangerment. However, I also presented evidence that the fragmented distribution has historical origins. Eastern and western *O. grande* populations were highly genetically divergent, indicating that they have long independent evolutionary histories that predate human settlement in New Zealand. This pattern most likely results from vicariance associated with Pleistocene glacial cycles. This finding does not diminish the vulnerable status of *O. grande*. Rather, it may be used to guide the identification of conservation units on the basis of evolutionary distinctiveness.

What caused the recent decline?

Although there are many candidate threats to *O. grande*, none have unequivocally been shown to have played a role in the species' decline (Whitaker & Loh, 1995). I tested a prominent existing hypothesis explaining the decline of *O. grande*: that clearing of native tussock grasslands has altered the metapopulation dynamics of the species (Whitaker, 1996). To do so, I genotyped skinks with microsatellite DNA markers (Chapter 2), and combined this information with demographic data to investigate connectivity of grand skink populations in pasture and tussock grassland dominated landscapes.

A novel feature of this study was the use of existing mark-recapture data to verify the accuracy of the genetic measures of dispersal (Chapter 3). I showed that skink populations were insular because skinks typically dispersed short distances (Chapter 4), and that this lead to higher levels of close inbreeding than has been documented previously in reviews of mammal and bird mating systems (Chapter 5). Yet, despite this, deleterious inbreeding effects were not obvious in *O. grande*, suggesting that such effects are currently unlikely to be important to population viability.

Significantly, I showed that the nature of the vegetation matrix surrounding skink populations both quantitatively and qualitatively affected grand skink dispersal dynamics (Chapter 4). Although the dispersal characteristics of individual populations varied, populations were on average more insular in pasture than in tussock dominated landscapes. Further, while female-biased dispersal was a feature of populations in tussock, no sex-biased dispersal was evident in pasture. I argued that the insularisation of skink populations in pasture is likely to make them more vulnerable to local extinction, and that replacement of tussock grasslands by pasture is a likely agent of decline for this species.

What can be done in light of these results?

It is difficult to be optimistic about the future prospects for *O. grande* while the processes that have driven the decline remain. The majority of montane tussock grasslands in central Otago have been cleared (W. Lee, Landcare Research, pers. comm.), and the process still continues. The best conceivable management outcome for grand skinks would be for tussock clearance to cease in areas where skink populations

occur. There are obvious difficulties with implementing such a plan because many skink populations occur on private land (G. Loh, pers. comm.). The reservation of tussock grasslands inhabited by skinks will therefore be an essential component of any conservation plan, and has already been partially implemented (Whitaker & Loh, 1995; Whitaker & Houston, 2002). This notwithstanding, species declines rarely have a single cause (Caughley & Gunn, 1996), and other factors, such as predation by feral carnivores, are also likely to be important, but their impacts are inadequately understood. Clearly, obtaining quantitative information on the impact of pest species must be a high priority. In addition, management would benefit from understanding the relative importance of different threatening processes. Spatially explicit simulation modelling of population dynamics under ecologically realistic scenarios may prove informative (e.g. Hokit *et al.*, 2001), as would the incorporation of sensitivity analyses (Mills & Lindberg, 2001). This study has supplied much of the data needed for such complex modelling (e.g. landscape-specific and sex-specific dispersal rates), and, in combination with the details of grand skink biology already known (e.g. Cree, 1994; Whitaker, 1996; Coddington & Cree, 1997; Tocher, 2003), provides a strong basis for the management of this species.

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Appendix I*

Making a microsatellite DNA library by hybridisation enrichment

Acknowledgements – This protocol is derived from one described by Tania King, which came originally from that of Armour *et al.* 1994 (*Human Molecular Genetics* **3**:599-605). I Thank Niccy Aitken, Leon Huynen, Hilary Millar, Pete Ritchie, Gwilym Haynes, Mathew Chan and Jenny Hay for assistance.

"Let time work for you"
Peter Ritchie, Palmerston North, 2000

Most of the reagents required for library construction are listed on page 141.

1. Start by making probes

Choose repeat motifs.
I would suggest tetranucleotides because when it comes to genotyping, your job will be a lot easier. Most people opt for dinucleotides because they are supposed to be more common. I had no trouble finding enough tetranucleotides for my study (10 loci). It is little extra work to try multiple motifs because they can be run simultaneously. I'd suggest trying a number of different ones, maybe both di- and tetranucleotide.

Order oligonucleotides.
I ordered $(AAAG)_7$, and its match $(CTTT)_7$ from Sigma-Genosys, also $(GATA)_7$/$(TATC)_7$. They cost about $50 a pair.
DNA, particularly single stranded DNA like these oligonucleotides should be stored as high pH solutions –not water! Best is TE pH 8.0.

The Oligos from Sigma came dehydrated. I make up to a standard concentration of 1 nmol/µl. This is the stock solution. I then made a working solution of 10 pmol/µl (=10uM) as a working stock. So that's a 1/100 dilution from the stock –I used water to make the dilution. Oligos get stored in the –20°C freezer.

Make lig-concatemers
i.e. Join the oligonucleotides together to make longer oligonucleotides.

1. *Phosphorylation* (add a phosphate group to the end of the oligo to facilitate ligation to others) and *annealing* (join the strands to their complement).
For each pair of oligos combine the following in a tube that will fit a PCR machine (e.g. 0.5ml):
2ug of each oligo
Ligase buffer (to 1x buffer overall)

* This appendix is not intended for examination, but is included to provide a complete record of laboratory protocols.

MilliQ water to 50 µl

Heat to 70°C for 5 minutes –use PCR machine.
Chill on ice (until chilled –a couple of minutes).
Add 3-5 µl T4 PNK. –this adds a phosphate group to the 3' end so that the oligos will ligate to one another (there is a free -OH group on the 5' end).
Incubate at 37°C for 45 minutes
Heat denature at 65°C for 20 minutes

2. *Now ligate them together…*
The 50 µl annealed oligos from before
Ligase buffer (to 1x buffer overall –including that already in the mix)
T4 DNA ligase 1µl.
MilliQ water to 65µl total

Incubate overnight at 16°C. (eg. put a waterbath in a 4 degree room)

3. *Isolate these 'lig-concatemers'* by size fractionating them from a *ca.* 1.2% agarose gel.
Use one large well per probe for the 65µl ligation mix (about 3 standard sized wells combined with tape) and gently fill. Run a 100bp ladder down each side of the main well. Don't run the gel too long –you want to minimise the amount of gel you have to cut out –just long enough so that you can see >200bp. Low voltage is good –about 100v.
Good practice: Put saran wrap on the UV transiluminator so you don't contaminate your DNA. With the UV on, quickly make marks where the DNA >200 bp is. Turn the UV off and cut out DNA >200bp with a sterile blade. Recover the DNA from the agarose using a kit (eg. Roche Highpure).

4. *Make giant probes* by 'primer-free' PCR. Do about 5 replicates for each probe (i.e. AAAG/TTTC and GATA/TATC)

1 x Buffer
6.7mM (!) $MgCl_2$
0.8mM dNTPs
2u Taq
1µl lig-concatemer template
to a total volume of 25µl.

For an I-cycler try a PCR cycle of: 95°C for 2 mins, [55°C for 20 secs, 72°C for 30 secs, 95°C for 30 secs] x 30, 72°C for 5 mins. Tanya used 55°C for 1 min, 72°C for 2 min, 95°C for 1 min on the old Hybaid Omni-E machine. A greater yield may be achieved with lower annealing temperatures.
Run out 2 µl or so of each. You should see a big smear out of the wells to ~100bp.

Pool all the replicates and ethanol precipitate. Resuspend on ~ 50µl milliQ water. Quantify. I got ~ 2ug/µl. Dilute to 1ug/µl.

Put them in the fridge.

Next,

2. Prepare your animal DNA

Extract DNA from about 5 individuals (both sexes if you don't know the heterogametic sex, or just use the heterogametic sex – if lucky you may find a sex marker). Digest each separately with NdeII (=MboI=Sau3A).
5µl genomic DNA
1 x buffer
1µl enzyme
0.5 µl RNAse
milliQ water to a total vol of 15 µl.

Digest overnight at 37 °C. An extra 0.5 µl of enzyme can be added later in the digestion.

Quantify the DNA from each digestion.

Combine these samples (equally) to get a total of 5-10ug DNA. Run the DNA out on a ~1% agarose gel (same procedure as above) and cut out DNA between 300 and 800 bp, again minimising the amount of gel to cut out. Tanya cut out 300-600bp, but I found that my microsats often didn't have much flanking sequence on one side –that may have been because they were tetras and very long. Recover the DNA from the agarose. Quantify it. I usually got between 10-40ng/µl.

Make 'SAU linkers'
Order the two halves and make up working concentrations with TE (I made it 1 nmol/µl)
SAULA (5' –GCGGTACCCGGGAAGCTTGG-3')
SAULB (5' –GATCCCAAGCTTCCCGGGTACCGC-3') (GATC is the overhang).

You want 5ug of SAULB and an equimolar amount of SAULA. Based on a 1nmol/µl working stock of SAULB, you will need 0.689 µl to make 5ug (a 1nmol/µl SAULB = 7.26 ug/µl). That's 0.689 nmoles and you'll also need 0.689 µl of SAULA.

To make the linker:

0.689 µl SAULA
0.689 µl SAULB
1.0 µl React 1 buffer (Gibco)
7.62 µl Q (a total volume of 10µl).

Make a 5 or 10 x master mix so you have replicates and the pipetting is more accurate.

Cycle through the following:

95°C - 40 secs
65°C – 15 min
60°C –15 min
55°C –15 min
50°C – 15 min
45°C – 15 min
40°C – 15 min

hold at room temperature.

Quantify it. I got ~1.12ug/µl (you put in about 10ug in 10µl).

Ligate the linkers onto the DNA
Ligate *ca.* 200ng DNA to 2.75 ug linker (a 1:250 molar ratio).
2.75ug SAU linker
200ng DNA
1 x ligase buffer*
1.0 µl T4 ligase
milliQ water to a total volume of 40 µl.

Incubate overnight @ 16°C in a waterbath.

* Ligase buffer contains ATP, which is sensitive to freeze-thaw. If you suspect it is degraded add 2µl of 10mM ATP to the ligation.

Put the ligation mix through a PCR purification kit to get rid of the unligated linkers (I used Highpure by Roche). Quantify. I got between 12.5 and 185ng/µl.

Pre-enrichment PCR (Tanya suggested that if you use a lot more DNA in the previous steps that this step can be left out. I did a pre-enrichment PCR, but fewer PCR steps could be better because PCR errors usch as recombination may be less likely).

1x Buffer
3mM MgCl2
0.8mM dNTPs
0.5um SAULA
10ng DNA template
1 U *Taq*
MilliQ water to 25 µl.
For the I-cycler I cycled through: 95°C –5min, [58°C – 30 sec, 70°C – 20 sec, 95°C – 30sec] x 30, 70°C - 4 min. On the Hybaid try: : 95°C –5min, [58°C –1min, 70°C – 2min, 95°C – 1min] x 30, 70°C - 4 min. In hindsight, I would try a gradient of annealing temperatures and probably use a lower annealing temperature.

Run out ~5µl from each reaction on a gel. You should see a smear between 300 and 800bp. Pool the reactions and ethanol precipitate them or put through a PCR purification column, resuspending on ~20µl. This is the size-selected DNA template that you will enrich from. Quantify. I got 420-792 ng/µl. You may need to pool multiple pre-enrichment runs.

3. Enrichment

Make some micro-membranes
Procure a small piece of membrane (eg. Gelman)
Being careful to avoid contamination cut out 30 or so squares about 2.5mm x 2.5mm. Store them in a 1.5ml tube.

Denature the probes
1ug probe

1µl 2M NaOH
1µl 20mM EDTA
milliQ water to 9µl

Set up 10 replicates for each probe and incubate them at 37°C for 30 minutes.
Neutralise with 1µl 1M Tris HCL pH 4.8, incubate for 10 minutes at room temperature.

Meanwhile, get a light table and put a couple of bits of gladwrap on it -label one for each probe with a permanent marker. Lay out 10 micro-membranes on each sheet.

Dot 1-2µl probe at a time onto each micro-membrane. You don't want the probe to run off the membrane so don't do too much at a time and let them dry before the next round. This is tedious. The light table is to speed up the drying process –its optional and you could use some other warm surface. You are aiming to put 1µg of probe per micro-membrane.

Once the membranes are all dried, fold gladwrap over the top and expose to the UV transilluminator for *ca.* 50 seconds per side. This is intended to crosslink the probes and bind them to the membrane. Store these in the –20°C freezer until use.

Hybridisation

Two options: you could run the hyb with both probes + DNA in the same tube, or each probe + DNA in separate tubes. It may not make a difference unless you definitely want a specific microsatellite motif. You should also run an extra tube(s) with membranes but no DNA added as a pseudo hyb-control.

Make 1300 µL of Church and Gilbert solution + 1% BSA for each tube you will run. Use a stock C&G soln. but add the BSA fresh (i.e. 10mg BSA powder per 1mL C&G soln.).
Pre-hyb membranes in 1ml of C&G/BSA at 65°C for 3+ hours.

After a couple of hours....

Denature your DNA in the same way as the probe.
1ug DNA
1µl 2M NaOH
1µl 20mM EDTA
MilliQ water to 9µl

Incubate at 37°C for 1 hour
Neutralise with 1µl of 1M Tris HCL pH 4.8, incubate for 10 minutes at room temperature.

Draw out the pre-hyb mix with a pipette leaving the membranes. Add 200 µL fresh C&G/BSA to the denatured DNA, mix and then add the mixture to membranes.

Hyb. overnight at 65°C rotating.

Washing

Note. Originally I processed my hybs and the hyb control at the same time. I spent a long time puzzling over the source of contamination in the hyb-control. It came from

the true-hyb - so its worth being fastidious and taking your time (the DNA in the hyb is PCR product so its very easy to contaminate). Do the whole washing procedure for the hyb-control before you start on the true-hyb. Also, make sure you spin the tubes down between each step. In hindsight, there is actually not much point in having the hyb control unless there have been a lot of libraries constructed in your lab with the same SAU linkers etc. It controls for contamination with SAU linkers but not much else.

Draw out the hyb liquid. Wash 3 times (at 65°C /rotating) for 10 minutes with 600 µl pre-warmed 2 x SSC/0.1 % SDS. Rinse at room temperature with 5 x SSC (no SDS). Draw out the liquid and air dry the membranes in the tubes.

Recover DNA
Add 100µl 50mM KOH/0.01% SDS. Stand with occasional flicking at room temperature for 10 minutes. Add 100µl 50mM Tris HCL pH 7.5/0.01% SDS. Flick a bit, take out membranes.

Precipitate DNA
1µl glycogen (20mg/ml) (this acts as a carrier for the DNA)
20µl 3M NaOAc pH 5.2
500µl 100% ETOH
Chill 10 minutes, spin 30 mins, wash with 500 µl 70% ETOH, spin again, dry, resuspend on 6µl milliQ water.

Amplify the enriched DNA (x 6)
1x Buffer
3mM MgCl2
0.8 mM dNTPs
0.5 um SAULA
1 U Taq
1 µl BSA
1µl DNA
MilliQ water to 10µl.

For the Hybaid: 95°C - 5 min, [67°C – 1min, 72°C – 1min, 95°C – 1min] x 35, 72°C – 4min. Alter it for the I-cycler –maybe 30 secs per stage. I am not sure why Tanya used such a high annealing temperature. A less stringent temperature would be better. Perhaps use the same temperature you found to be optimal in the pre-enrichment PCR.

Pool PCRs and size fractionate 300-800bp DNA on a 1.2 % agarose gel. Recover DNA with a kit. Minimise the amount of gel to cut out by running only for a short time. You should see a smear between 300-800bp.
If you did one, also run a PCR using hyb-control - there shouldn't be a product.
Digest the DNA with NdeII to get rid of the 'SAU-linkers' and leave GATC 'sticky ends'. Incubate at 37°C for 2 hours. Deactivate enzyme by heating for 20 mins at 65°C. Put through a PCR purification kit to get rid of the linkers. This is your insert DNA. Quantify. I got 20 ng/µl. Having a good amount of DNA is v. important here –I ended up pooling the results of 2 hybs and ethanol precipitating them.

4. Cloning

Ligation of insert into a pUC18 plasmid vector cut with BamHI

Work out the appropriate quantities of DNA and plasmid to get the right molar ratio (insert:plasmid). I had success with a molar ratio of 7:1 and 10:1, but Tanya used 3:1.....

e.g. $\frac{\text{Ave. size insert}}{\text{Size pUC18}} \times \frac{7}{1} \longrightarrow \frac{550}{2700} \times \frac{7}{1} = 1.43$

I used 50ng of pUC18 in the ligation, so you would need 1.43 x 50 ng of insert (= 71.5ng).
1x ligase buffer
1µl T4 DNA ligase
pUC18/BamHI
Insert DNA
MilliQ water to 10µl

Incubate overnight @ 16°C. Don't heat de-activate.

Transformation of ligated plasmids into cells

First, make agar plates –ten per transformation and let them cool fully (see appendix).

I used DH5α cells from Lifetech and pretty much followed their instructions.
Dilute the ligation mix 5-fold with TE (pH 7.5).
Put 100µl cells in a 10 ml tube, and add 1.5µl ligation mix, moving the pipette gently while dispensing.
Incubate on ice for 30 minutes.
Heat shock in a water bath @ 42°C for 45 seconds.
Place on ice for 2 minutes.
Add 900µl SOC buffer, place on shaker for 1 hour (or a bit longer) @ 225 rpm @ 37°C. The mix should go a bit cloudy.
Spread 100µl of transformation mix evenly onto each plate. When dry, put lid on, turn upside down and allow to grow overnight at 37°C.

Colonies containing recombinant plasmids will show up white and non-recombinant blue. When the colonies are big enough to identify colour, pick them into a microtitre plate (see below). Putting the plates at 4°C can help the colour come up.

Medium for picked colonies
Microtitre plates have 96 wells and you'll need about 100µl per well (depends on the length of the hedgehog spikes).
80µl L-broth per well
20µl glycerol per well
0.1µl Ampicillin per well (@50mg/ml)

Leave the bottom right well empty for orientation. Use a new yellow tip to scrape a bit of each white colony, then swirl it in a well. Gently shake the plate for 3-6 hours at 37°C. Parafilm/tape the lid closed and store at –80°C until use.

Transferring colonies to a membrane (Hedgehogging)
Cut a piece of membrane to fit the microtitre plate. Cut a separate membrane for each probe you intend to use. Cut off the bottom right corner and label the top left with pencil. Lie the membranes on a large plate containing L-agar with ampicillin at 100ug/ml. Prepare a bath of 100 % ethanol for sterilising the hedgehog (use a microtitre plate lid). Dip the hedgehog into ethanol, flame, then allow to cool. Dip cool hedgehog into thawed colonies in microtitre plate for a few seconds. Transfer to membrane, keeping the orientation the same –pressing gently to make sure all the spikes contact. When all done, put the lid on or cover large plate with alfoil and grow overnight at 37°C. If you don't have a hedgehog, you can do this step manually with yellow tips.

The next day you should see small yellowish colonies where the hedgehog contacted the membrane.

Lyse the colonies onto the membrane
Use forceps to place the membranes onto a piece of filter paper that has been pre-wetted with 2 x SSC/5% SDS for 2 minutes. Remove the membranes and microwave them on high for about 40 seconds or until dry (not burnt). This is best done on a clean paper towel. Wrap in gladwrap and store at room temp until use.

5. Radioactive Probing

Wash the membrane in 2 x SSC. Float for 2 minutes then sink and soak for a further 5 minutes.

Prehybridisation
For each probe make:
75ml 0.5M Na_2HPO_4
75mls MilliQ water
300µl EDTA
10.5g SDS (add last and wear a mask).
Warm it in the microwave to dissolve the SDS (about 1.5 min on medium).

Pour into your plastic Tupperware probing container. Put membranes in and pre-hyb for 1.5 hours at 65°C with v. low rocking. Also put in a small piece of blank membrane as a control. You can put multiple membranes on top of one another in a box –just make sure they get covered with the hyb mix.

Meanwhile,
Make your radioactive probes
I made these with the 'giant probes' and another time the 'lig-concatemers'. In the Armour et al paper, they found that large probes were more effective at revealing microsatellites than the standard 30mer oligos that people often use.
Dilute the giant probes or lig-concatemers probes to 5ng/µl.
You need a labelling kit (eg. Megaprime kit from Ampersham).
Put 5µl of each giant probe into labelled 1.5 ml tubes plus 5µl of primer from the megaprime kit.
Denature by floating in boiling water for 5 minutes. Spin down.
Add 10 µl labelling buffer

Add 2µl klenow enzyme
Add 25µl milliQ water (total volume is now 50µl). Mix by flicking and spin down.

Behind screen add 3µl isotope to each tube and mix by pipetting (add more isotope if it's older).
Put the sample in a pre-warmed (37°C) lead pottle and incubate at 37°C for 1 hour.

Meanwhile,
Make columns (to use to separate unincorporated isotope).
You need 2 x 10ml tubes and, 2 x 1ml syringes without plungers per probe. Also, siliconised beads for each syringe.
Get a bead to sit at the bottom of the syringe, and the syringes to sit in the 10ml tubes. Use a disposable pipetter to fill the syringe with sephadex –avoiding air bubbles. When full, spin at ~2250 rpm for 4 minutes. Throw away the flow through and refill syringe with sephadex. Repeat this until only sephadex granules fill the syringe to 1ml. The columns are now ready.

Retrieve probe and add 50µl TE and 5µl 0.2M EDTA to each. Flick and spin down,

Put a lidless 1.5ml tube in the bottom of the 10ml tube. Load the probe into the top of the first column and spin @ 2250rpm for 4 minutes. Throw out syringe.
Pipette the flow through into the next column and repeat spin.
Pipette the flow through into a new, labelled 1.5 ml tube with a pierced lid. Float on boiling water for 5 minutes (in a fume-cupboard with a screen), then put on ice for 5 minutes.
Add the probes to the hyb boxes and leave overnight at 65°C with v. gentle rocking. A Geiger-counter set at 100x should indicate that the boxes are HOT. Make sure the boxes are well sealed.

Rinsing membranes
Make a 2x SSC/0.1% SDS wash solution. Warm it to 65°C. When ready, pour the probes into schott bottles and store behind a screen. Put the oven to 50°C. Pour wash solution into container and membranes and swish gently around for ca. 30 secs. Tip down the sink with plenty of water. Repeat. Pour remainder of wash solution into containers and put back in oven @ 50°C for 15 minutes.
Tip wash down the sink. Check the blank membrane with the Geiger counter on low setting (0.1x) –it should be blank. Pat membranes dry.

Prepare membranes for film
On a cutting board behind a screen wrap each membrane in saran wrap separately and avoiding airbubbles. Leave about 1cm saran wrap around edges. Lie the membranes colony side up in a x-ray cassette with a screen. Map the positions of membranes. When all ready, take cassette and box of film to dark room.
Under red light cut bottom right corner of film for orientation. Lay the film over the membranes. Close and put in -80°C freezer overnight.

Next day, put the cassette in 37°C room until it thaws out.

Develop the film
Under red light fix clips to one edge of the film. Dip it into developer and shake gently for 3 minutes.
Wash with water

Dip into fixer for 2 minutes
Wash with running water for ~10 minutes and hang to dry. Lights on. You should see black dots where positive colonies are.
** On average, one third of my colonies came up positive for microsatellite inserts.**

Growing and sequencing positives
Use a grid to work out which colonies are positive. Thaw out the microtitre plate and swirl a yellow tip in each positive. Drop the tip into a 10ml tube containing 3mls of L-broth with 0.1mg/ml ampicillin. Put alfoil over the top (allow air to circulate). Grow with 500rpm shaking at 37°C overnight. Also run a tube containing a blank yellow tip.

Miniprep and sequence positive clones.

Appendix.

Useful solutions.

20 x SSC
175.3g Sodium chloride
88.2g Sodium citrate
Make to 800ml and pH with 10M NaOH.
Fill to 1 litre.

Church and Gilbert solution
75ml 1M Disodium hydrogen orthophosphate
300µl 0.5M EDTA
10.2ml 10% SDS
64.5ml milliQ water

SOC buffer
2g bactotryptone (= bactopeptone)
0.5g bactoyeast
1.0ml 1M NaCl
0.25ml 1M KCl
1ml 2M $Mg2^{+}$ stock solution (filter sterilised)
1ml 2M glucose (filter sterilised)

Mix the first 4 ingredients and fill to 98mls with milliQ water. Autoclave and cool.
Add Mg^{+} and glucose.

L-Broth (L-agar is the same but add 15g agar –and mix on heat to dissolve)
10g peptone
5g yeast extract
10g NaCL
make up to 1L with dH_20
Divide into 300ml aliquots and put each into a 1L bottle.
Autoclave.

0.5M EDTA pH 8.0

18.61g EDTA
80ml milliQ water
pH to 7.0 with NaOH pellets
then pH to 8.0 with 10M NaOH solution
Make up to 100mls and check pH

A list of chemicals/enzymes required

- [ ] *Taq*, $MgCl_2$ PCR buffer and dNTPs
- [ ] Enzyme (NdeII=MboI=SAU3A)
- [ ] Agarose etc
- [ ] Linker DNA (order these)
- [ ] T4 DNA ligase (plus buffer)
- [ ] Oligos of the chosen repeat motifs (order these)
- [ ] T4 PNK (= phosphonucleokinase)
- [ ] Gel DNA recovery kit (eg. Highpure -Roche)
- [ ] 2M NaOH
- [ ] 20mM EDTA
- [ ] 1M Tris HCL pH 4.8, also pH 7.5
- [ ] Church and Gilbert solution
- [ ] 20 x SSC buffer
- [ ] 10 % SDS
- [ ] 1M KOH
- [ ] Glycogen
- [ ] NaOAc pH. 5.2
- [ ] 100 % ethanol, 70% ethanol
- [ ] pUC18 plasmid cut with Bam H1 and cap'ed
- [ ] Max efficiency DH5α cells (eg. Gibco).
- [ ] SOC buffer
- [ ] L-broth/L-agar
- [ ] Ampicillin
- [ ] Glycerol
- [ ] 0.5 M Na_2HPO_4
- [ ] Isotope, film, labelling kit, sephadex to make columns (for radioactive work - if applicable)