The role of genetics in kakapo recovery

BRUCE C. ROBERTSON
School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand.
bruce.robertson@canterbury.ac.nz

Abstract A growing literature indicates that genetic factors have a significant impact on the persistence of populations and hence play an important role in species recovery. Here, I review the role of genetic research in the recovery program of the critically endangered kakapo (*Strigops habroptilus*). By using three examples of how genetics has guided kakapo managers (molecular sexing, quantification of genetic diversity and confirmation of paternity from known matings), I highlight the important contribution genetics has made to kakapo recovery. I also explore three new avenues of research (genetic diversity at genes for disease resistance, molecular ageing, and genetic similarity and hatching success), all of which may have important implications for future conservation management of kakapo. As such, this review demonstrates that genetic research is an integral part of kakapo recovery.

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INTRODUCTION

Inbreeding, genetic drift and the loss of genetic variation are inevitable consequences of small population size (Frankham *et al.* 2002; Keller & Waller 2002; Frankham 2005). Although some authors have questioned the importance of genetic factors in conservation (Caro & Laurenson 1994; Caughley 1994), a growing literature suggests that such factors have a significant impact on the persistence of contemporary populations, and hence future population size (Spielman *et al.* 2004; Frankham 2005). For example, genetic rescue (i.e., the introduction of genetic variation into small inbred populations via translocations: Ingvarsson 2001) of greater prairie chickens (*Tympanuchus cupido pinnatus*) and adders (*Vipera berus*) reversed declining populations by improving egg hatchability and reducing the occurrence of stillborn offspring, respectively (Westermeier *et al.* 1998; Madsen *et al.* 1999). Similarly, in a Scandinavian population of grey wolves (*Canis lupus*) new genetic variation introduced from a single migrant appears to have stimulated exponential population growth (Vila *et al.* 2003).

Although the theory underlying the application of genetics to conservation issues (i.e., conservation genetics) has advanced dramatically over the last 20 years (Hedrick & Miller 1992; Frankham *et al.* 2002), incorporation of these advancements into endangered species recovery plans has been limited (Stinchcombe *et al.* 2002). A recent examination of 181 US endangered species recovery plans from 1977 to 1998 found that, although genetic threats to species recovery were identified in almost 25% of recovery plans, genetics only played a minor, often ill-defined, role in conservation management strategies (Moyle *et al.* 2003). The early recovery program of the kakapo (*Strigops habroptilus*) was no exception, but in the last 10 years greater focus has been placed on genetics in kakapo management.

Kakapo recovery only became possible with the discovery of female kakapo on Stewart Island in 1977, as prior to this the species consisted of a handful of males from Fiordland (Merton *et al.* 1984; Powlesland *et al.* 1995). Beginning with the initial recovery plan (Powlesland 1989), kakapo managers have placed importance on the maintenance of genetic diversity in the species' recovery. However, rather than quantifying the levels of variation within kakapo – a more logical first step – managers proposed to achieve this goal by the collecting and storaging sperm from the remaining Fiordland kakapo for future artificial insemination (Powlesland 1989). In a subsequent review of the kakapo recovery program, Imboden *et al.* (1995) identified explicit goals to conserve genetic variation: recommendation 48 called for the relatedness between all kakapo be determined "using appropriate DNA techniques, e.g. microsatellite fingerprinting" and introduced the idea of "genetic management of the kakapo population". This recommendation was subsequently incorporated into the 1996-2005 kakapo recovery plan (i.e., Objective 5, maintain genetic diversity: Cresswell 1996).

The first study of genetic variation in kakapo provided an indirect measure of DNA variation. Triggs *et al.* (1989) assessed levels of allozyme (i.e., protein) variation and found that Stewart Island kakapo had similar low levels of allozyme variation as found in most birds (see Frankham *et al.* 2002). However, allozymes are a poor reflection of the overall variation in an organism's genome because DNA-level mutations may not result in detectable differences in protein structures (DeWoody & Avise 2000). Furthermore, allozymes tend to have low levels of variability (DeWoody

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& Avise 2000) possibly because of their functional basis (i.e., enzymatic) and hence the influence of natural selection (Avise 2004). Current approaches to quantifying levels of genetic variation in populations use molecular markers for putatively selectively-neutral regions of DNA, such as microsatellite and minisatellite DNA markers, which typically display high levels of genetic variation (Avise 2004). Using neutral markers, recent investigations into kakapo genetic variation have suggested that, contrary to Triggs *et al.* (1989) findings, Stewart Island kakapo do not display similar levels of genetic variation to most other birds, rather the population displays low levels of variation (Robertson *et al.* 2000b; Miller *et al.* 2003; see below).

Despite being hampered by low genetic variation, genetic research has made important contributions to the management strategy of kakapo. In this review, I examine the three significant ways that genetics has guided kakapo recovery: molecular sexing, quantification of genetic variation, and confirmation of paternity from known matings. I also highlight three avenues for future research that may provide important information for future management of kakapo.

MOLECULAR SEXING OF KAKAPO

Background

Knowledge of the sex of individuals is essential information in the study of any animal population and particularly relevant in species conservation. For example, avoiding same sex pairings in captive breeding programs is critical for endangered species recovery (e.g., kaki *Himantopus novaezelandiae*: Millar *et al.* 1997). However, many bird species are sexually monomorphic and hence are difficult to sex. For these species, the only reliable way to distinguish males and females is to determine sex using DNA tests (Ellegren & Sheldon 1997; Griffiths 2000).

Kakapo have proven difficult to sex using non-molecular means. Initial attempts to sex birds were hampered by the lack of known-sex individuals, particularly females (Reid 1969). Subsequent inclusion of known-sex individuals identified sexual dimorphism in body mass and some skeletal elements, with males tending to be larger than females, male-specific breeding behaviour (i.e., vocalisations and displays) and a possible sexual difference in plumage (Merton *et al.* 1984; Powlesland 1989; Higgins 1999; Robertson *et al.* 2000a). These sexual differences, however, are confounded by massive seasonal and annual fluctuations in weight (Elliott *et al.* 2001), and the lack of breeding by some kakapo (Powlesland *et al.* 1995; Clout & Merton 1998); hence uncertainty surrounded the sex of some adults. Importantly for current population management, no non-molecular method existed to determine the sex of dead embryos and dead nestlings, which account for a large proportion of kakapo productivity each year (Elliott *et al.* 2001, 2006).

Molecular sexing, when conducted with the appropriate rigour (Robertson & Gemmell in press), provides the most definitive test available of sex (see reviews in Ellegren & Sheldon 1997; Griffiths 2000). In birds, females are heterogametic (ZW) and males are homogametic (ZZ): detection of the female-specific W chromosome allows the sexes to be distinguished. A number of molecular sex tests have been developed over the last decade (Griffiths 2000), but the most commonly used of these employ the polymerase chain reaction (PCR: see review in Avise 2004) to detect fragments of the Z and W chromosomes with near universal success across species (Griffiths *et al.* 1998; Fridolfsson & Ellegren 1999). PCR-based sexing has the important advantage that it can be applied to minute amounts of potentially degraded DNA (Griffiths 2000), allowing sex to be determined from a range of tissue types including early death embryos, feathers, museum skins, carcass remains and faeces (e.g., Robertson *et al.* 1999; Robertson *et al.* 2000a; Clout *et al.* 2002; Robertson *et al.* in press).

Empirical findings

Kakapo sex is determined using the consensus of two independent PCR-based molecular sex tests (Griffiths *et al.* 1998; Fridolfsson & Ellegren 1999): both tests resolve length polymorphism at distinct regions of the same gene. By using a consensus approach, the well-known limitations of PCR amplification are accommodated (e.g., allelic dropout, preferential amplification and null alleles: Pemberton *et al.* 1995; Taberlet *et al.* 1996; Gagneux *et al.* 1997; Dawson *et al.* 2001), hence greatly reducing the chance of incorrect identification of sexes (Robertson & Gemmell in press).

Since 1996, molecular sexing has been used to sex all surviving and recently deceased adult kakapo (including seven specimens held in the Museum of New Zealand Te Papa Tongarewa) and 69 fertile kakapo eggs (Robertson *et al.* 2000a; B. Robertson unpubl. data). During breeding years, molecular sexing has allowed kakapo managers access to the sex of chicks often within days of hatching by using blood spots from egg shells (B. Robertson unpubl. data). High embryo and nestling mortality (Elliott *et al.* 2001), however, means that molecular sexing is the only way to determine sex for many kakapo eggs (e.g., 41% of the 69 known-sex kakapo eggs required molecular sexing: B. Robertson unpubl. data).

The most significant contribution of molecular sexing to kakapo recovery has been the discovery that *ad libitum* supplementary feeding (Elliott *et al.* 2001) was producing an undesired male bias in productivity (Clout *et al.* 2002). This was not a new idea to kakapo managers (B. Robertson unpubl. data), but it became a priority with the publication of an independent commentary on the potential impact of supplementary feeding on kakapo sex ratios (Tella 2001). Had managers failed to detect the male bias, which emulated the natural male bias in the extant population (Clout & Merton 1998; Robertson *et al.* 2000a; Elliott *et al.* 2001, 2006), kakapo recovery could have been setback

by between 12 to 109 years depending on the level of breeding management (i.e., all females to no females intensively-managed)(Robertson *et al.* in press).

Molecular sexing was pivotal in relating kakapo sex ratios to supplementary feeding because of the high mortality of kakapo embryos and nestlings (Elliott *et al.* 2001; Clout *et al.* 2002). Of the 33 individuals from 18 clutches sexed, 12 (36%) could only be sexed using molecular tests of sex. Exclusion of these embryos and dead nestlings would have seriously limited the power of the sex ratio analysis by reducing the number of broods to 13 and increasing the proportion of single chick clutches from 33% to 54%. The discovery that female kakapo in better condition (supplementary fed *ad libitum*) were producing more sons resulted in changes to supplementary feeding which removed the male-bias in subsequent breeding (Robertson *et al.* in press).

Additional outcomes

Molecular sexing has found novel application in kakapo recovery, with the kakapo being the first bird to be sexed from faeces (Robertson *et al.* 1999). Faecal sexing was developed in kakapo in order to assign fecal samples to males or females so that sexual differences in diet could be investigated, with a view to identifying triggers of breeding in kakapo (G. Elliott pers comm.; Wilson *et al.* 2006). After considerable research and development, a methodology was defined that returned 100% success in sexing fresh faecal samples (<1 day old) from known-sex kakapo (Robertson *et al.* 1999). Unfortunately, success was much lower in the target faecal samples: 39% success in 41 faecal samples of indeterminate age collected between 1977 and 1999. Consequently, kakapo managers discontinued the molecular sexing of faeces. The reason for the lower success in sexing older faeces is unknown but could be due to loss of epithelial cells during weathering. Recent genetic studies using possum faeces as a DNA source found that weathering limited the usefulness of faeces to less than 28 days from defecation (D. Gleeson pers. comm.).

Molecular sexing also has confirmed the sexual nature of the subtle plumage difference of the primary wing feathers of kakapo (Powlesland 1989; Higgins 1999; Robertson *et al.* 2000a). Male kakapo show a pale yellow-brown barring or mottled pattern on the distal 3-4 cm of the inner vane of each of the four outermost, full-length primaries (primary number 6 to 9), whereas females lack this pattern (Robertson *et al.* 2000a). Using molecular sexing, Robertson *et al.* (2000a) verified the sexual nature of this plumage pattern in both extant adult (50 individuals) and fledgling (13 individuals) kakapo and also in extinct mainland populations sampled using museum skins (17 individuals) collected over 100 years ago. Molecular verification of the sexual nature of the plumage polymorphism provides a reliable and rapid test of sex for kakapo in the field, especially as new individuals are occasionally discovered (e.g., in 1997 on Stewart Island). The plumage sexual difference also provides an inexpensive method to increase the value of museum skins lacking any collection details, which includes a considerable proportion of the skins in museum collections the world over (Williams 1960; Reid 1969; B. Robertson unpubl. data).

QUANTIFYING GENETIC DIVERSITY IN KAKAPO

Background

Endangered species typically possess less genetic variation than closely related non-endangered species (Frankham *et al.* 2002). This is because endangered species, by definition, have smaller population size and genetic variation is lost faster due to genetic drift (i.e., the random sampling of gametes between generations) in smaller populations: the rate of loss per generation due to genetic drift is $1/2N$, where N is the census population size (Frankham *et al.* 2002). Furthermore, inbreeding (i.e., breeding between close relatives), which also negatively impacts on genetic variation, becomes increasingly unavoidable in smaller populations (Frankham *et al.* 2002).

A key goal of kakapo recovery is the maintenance of remaining genetic diversity (Objective 5: Cresswell 1996). This is an important goal because loss of genetic variation can increase susceptibility to environmentally inflicted mortality (Keller & Waller 2002), reduce the overall fitness of populations (e.g., Westermeier *et al.* 1998; Madsen *et al.* 1999) and suppress population growth thereby slowing species recovery and prolonging the threat of extinction (e.g., Vila *et al.* 2003). However, before management strategies can be established to retain genetic variation, managers need knowledge of the genetic diversity present in the extant kakapo.

In kakapo, two lines of anecdotal evidence suggest that the remaining kakapo might possess low levels of genetic diversity. First, all but one of the extant birds originated from Stewart Island (Powlesland *et al.* 1995; Clout & Merton 1998). Island populations generally contain lower genetic variation than mainland populations due to smaller population sizes (Frankham 1997; Frankham *et al.* 2002). Second, consistent with a lek mating system (Höglund & Alatalo 1995), kakapo display strong variance in male reproductive success (Miller *et al.* 2003). With only a small proportion of males contributing their genes to subsequent generations, genetic variation will be further reduced in the population. The potential for low levels of genetic variation in kakapo highlights the importance of understanding what variation remains in kakapo and also the need to identify genetically distinct non-breeding individuals which should be included in the breeding population.

From the perspective of conservation, one of the most important forms of genetic variation is that which impacts on reproductive fitness and hence the persistence of populations. Unfortunately, this type of variation is the most difficult to examine because variation in these

Table 1 Ninety-one microsatellite loci screened for genetic variation in extant kakapo

Microsatellite locus	No. kakapo	Amplification#	Species of origin	Reference
*Sha*1	86	P	Kakapo, *S. habroptilus*	Robertson *et al.* 2000b
*Sha*2, *Sha*3, *Sha*4, *Sha*5, *Sha*6, *Sha*7	86	M	Kakapo, *S. habroptilus*	Robertson *et al.* 2000b
*Sha*8, *Sha*9, *Sha*10, *Sha*11	86	M	Kakapo, *S. habroptilus*	B. Robertson unpubl. data
ZL04, ZL12, ZL14, ZL18, ZL22, ZL35, L37, ZL38	51	Ø	Silvereye, *Zosterops lateralis*	Degnan *et al.* 1999
*HrU*3, *HrU*5, *HrU*6, *HrU*7	51	Ø	Barn swallow, *Hirundo rustica*	Primmer *et al.* 1996
*Ero*1, *Ero*2, *Ero*4, *Ero*5, *Ero*8, *Ero*11	86	M	Eclectus parrot, *Eclectus roratus*	Adcock *et al.* 2005
*Ero*3	86	P	Eclectus parrot, *E. roratus*	Adcock *et al.* 2005
*Nmed*17, *Nmed*36	86	P	Kaka, *Nestor meridionalis*	Sainsbury *et al.* 2004
*Nmed*20, *Nmed*21, *Nmed*31, *Nmed*33, *Nmed*43	86	M	Kaka, *N. meridionalis*	Sainsbury *et al.* 2004
*Nmed*8, *Nmed*11, *Nmed*19, *Nmed*23, *Nmed*24, *Nmed*26, *Nmed*32	86	M	Kaka, *N. meridionalis*	J. Sainsbury unpubl. data
*LEI*160	86	M	Chicken, *Gallus gallus*	Wardle *et al.* 1999
Hle0A10dd, Hle6A11, Hle6F07	2*	M	Bald eagle, *Haliaeetus leucocephalus*	Tingay *et al.* unpubl. data
Hle0B06, Hle0C03dd, Hle0E12, Hle6A09dd, Hle6A10dd, Hle6F02, Hle6F12, Hle6H10	2*	Ø	Bald eagle, *H. leucocephalus*	Tingay *et al.* unpubl. data
Hv00B01	2*	M	Madagascar fish eagle, *H. vociferoides*	Tingay *et al.* unpubl. data
Hv01G02, Hv01G04, Hv01H7dd, Hv019dd, Hv046dd, Hv055, Hv059, Hv068dd, Hv084, Hv088	2*	Ø	Madagascar fish eagle, *H. vociferoides*	Tingay *et al.* unpubl. data
Hal-01, Hal-03	2*	M	White tailed sea eagle, *H. albicilla*	Hailer *et al.* in press
Hal-05, Hal-07, Hal-09, Hal-10, Hal-13, Hal-15	2*	Ø	White tailed sea eagle, *H. albicilla*	Hailer *et al.* in press
Aa57	2*	M	Spanish imperial eagle, *Aquila adalberti*	Martinez-Cruz *et al.* 2002
Aa02, Aa04, Aa11, Aa12, Aa15, Aa26, Aa35, Aa36, Aa43, Aa49, Aa50, Aa53, Aa56	2*	Ø	Spanish imperial eagle, *A. adalberti*	Martinez-Cruz *et al.* 2002
IEAAAG15	2*	M	Spanish imperial eagle, *A. adalberti*	Busch *et al.* 2005

P = polymorphic amplification, M = monomorphic amplification and Ø = no amplification; * Cross-hybridisation of bird microsatellite primers was done by Deborah Dawson and Ruth Tingay and was assisted by Deborah Dawson's BIRDMARKER webpage maintained at the NERC-funded Sheffield Molecular Genetics Facility, UK.

so-called quantitative characters is under the control of multiple genes and also environmental causes (Frankham *et al.* 2002). Given the difficulty of measuring quantitative genetic variation, geneticists typically use genetic variation detected with selectively-neutral molecular markers as a proxy of genome-wide variation (see Avise 2004 for discussion of neutral markers in conservation).

Genome-wide genetic variation in kakapo has been surveyed with two independent classes of neutral molecular markers: minisatellite DNA fingerprinting and microsatellite DNA markers (Robertson *et al.* 2000b; Miller *et al.* 2003). Both classes of markers detect typically highly-variable repeated sequences of DNA. Minisatellite DNA fingerprinting detects variation in tandemly-repeated, conserved core sequences (10-15bp long) dispersed throughout an organism's genome and produces an individual-specific banding profile (a DNA fingerprint: Jeffreys *et al.* 1985), while microsatellite markers examine variation at short repeated sequences (usually di, tri, or tetra-nucleotide motifs: $(CA)_n$, $(CAG)_n$, $(CAGT)_n$) that are tandemly-repeated at a single position (i.e., locus) on a chromosome (Avise 2004). For microsatellites, genome-wide variation is examined by PCR amplification of a number of microsatellite markers, a process which has been termed microsatellite DNA fingerprinting.

Microsatellites are now the marker of choice for examining relatedness and population genetic structure, because, amongst other reasons, genetic variation can be assessed from minute amounts of DNA, such as from early death embryos and dead nestlings (*cf.* minisatellite DNA fingerprinting which requires large amounts of high quality DNA) (Avise 2004). One major drawback of microsatellite markers is that they tend to be species-specific, and hence need to be developed for each new species examined, although microsatellite markers developed for one species can amplify DNA in others (i.e., cross-hybridisation: Primmer *et al.* 1996; Robertson & Gemmell 2005).

Empirical findings

Genetic variation in kakapo was first examined at the DNA level using microsatellite markers specifically developed for kakapo (Robertson *et al.* 2000b). The remaining kakapo from Stewart Island showed no genetic variation at eleven microsatellite loci (Robertson *et al.* 2000b; B. Robertson unpubl. data). Only Richard Henry, the last surviving Fiordland kakapo, displayed any variation: one locus (*Sha*1; Robertson *et al.* 2000b) was variable (Miller *et al.* 2003; B. Robertson unpubl. data). Subsequently, a large number of bird microsatellite loci have been cross-hybridised with kakapo DNA and few polymorphic loci have been identified in extant kakapo (i.e., a further 3 of 34 loci screened with large numbers of individuals displayed variation: Table 1).

Table 2 Minisatellite DNA bandsharing coefficients for various threatened insular bird populations.

Species	Status*	Bandsharing (±SD)#	Minimum population size§	Current population size*	DNA probes	Bandsharing reference
Blue duck, *Hymenolaimus malacorhynchos*	Endangered	0.39 ± 0.05	2440	2440	33.15, 3'HVR, pV47-2, & per	Triggs *et al.* 1992; King *et al.* 2000
Puerto Rican parrot, *Amazona vittata*	Critical	0.44	13	44	Per & 33.6	Brock & White 1992
Seychelles warbler, *Acrocephalus sechellenis*	Vulnerable	0.5	50	2,060	33.15	Komdeur *et al.* 1998
Guam rail, *Gallirallus owstoni*	Extinct in wild	0.62	20	180	α 98 & Per	Haig *et al.* 1994
Takahe, *Porphyrio hochstetteri*	Endangered	0.68	120	15-220	33.6	Lettink *et al.* 2002
Shore plover, *Thinornis novaseelandiae*	Endangered	0.68 ± 0.21	159	159	pV47-2 & per	Lambert *et al.* 2000
Galápagos hawk, *Buteo galapagoensis*	Vulnerable	0.69 ± 0.08	400-500	400-500	33.15 & 33.6	Faaborg *et al.* 1995
Hawaiian goose, *Branta sandvicensis*	Vulnerable	0.70	30	960-1000	M13 & 33.6	Rave 1995
Auckland Is teal, *Anas aucklandica*	Vulnerable	0.71	600-2000	600-2000	pV47-2	Lambert & Robins 1995
Kakapo, *Strigops habroptilus*	Critical	0.71 ± 0.07	50	86	33.15, 33.6 & per	Miller *et al.* 2003
Mariana Crow, *Corvus kubaryi*	Endangered	0.77	20	220	33.6 & 33.15	Tarr & Fleischer 1999
Black robin, *Petroica traversi*	Endangered	0.83 ± 0.04	5	178	pV47-2, 33.6 & 33.15	Ardern & Lambert 1997
Campbell Island teal, *Anas nesiotis*	Critical	0.87 ± 0.04	9	120	pV47-2 & 33.15	M. Williams, D.M. Lambert unpubl. data

* BirdLife International website [http://www.birdlife.org/datazone/species/index.html]; # averaged across DNA probes where more than one bandsharing coefficient reported for unrelated individuals; § lowest number based on reported numbers on BirdLife International website.

Low levels of genetic variation at microsatellite loci are suggestive of a general lack of genome-wide variation in the surviving kakapo, which is consistent with the insular origin of all but one of the surviving kakapo and possibly the hypothesized negative impact of the species' lek mating system on genetic variation. Indeed, low allelic diversity at microsatellite markers has also been noted in other threatened species that all have small and/or insular population sizes (e.g., black-footed rock-wallaby *Petrogale lateralis*: Eldridge *et al.* 1999; Hawaiian monk seal *Monachus schauinslandi*: Kretzmann *et al.* 2001; musk ox *Ovibos moschatus*: Holm *et al.* 1999; but see van Coeverden & Boag 2004; San Nicolas Island fox *Urocyon littoralis dickeyi*: Aguilar *et al.* 2004). Investigation of the ancient DNA of kakapo from now-extinct mainland populations of kakapo also adds weight to this conclusion (Robertson *et al.* 2000b). For example, the kakapo-specific microsatellites show allelic variation in kakapo from a number of now-extinct mainland populations, including Dusky Sound, while Stewart Island birds display none (Robertson *et al.* 2000b; B. Robertson unpubl. data).

Genome-wide variation in kakapo has also been examined using bandsharing coefficients (i.e., proportion of bands shared between the DNA profiles of two individuals) calculated for putatively unrelated individuals in a minisatellite DNA fingerprinting study of kakapo paternity (Miller *et al.* 2003: see next section). Putatively unrelated kakapo originating from Stewart Island shared approximately 68% to 80% of their bands with other kakapo (Miller *et al.* 2003; Table 2). In comparison, the average for bird species is about 24%, with bandsharing coefficients greater than 0.5-0.65 deemed indicative of "a genetically depauperate population" (Papangelou *et al.* 1998). The surviving Stewart Island kakapo, therefore, appear to have low levels of genetic variation relative to other bird species that is likely attributable to prolonged genetic drift and inbreeding associated with small population size (Miller *et al.* 2003; Jamieson *et al.* in press).

Although Stewart Island kakapo possess low levels of minisatellite DNA variation, the level is not outstanding when compared to other threatened insular bird species (Table 2). This comparison shows a couple of interesting points: (1) kakapo have similar bandsharing coefficients as other threatened New Zealand birds: namely, takahe (*Porphyrio hochstetteri*), Auckland Island teal (*Anas aucklandica*), and shore plover (*Thinornis novaseelandiae*); (2) Also, genetic variation in Stewart Island kakapo is similar to insular species that may or may not have been through a recorded demographic population bottleneck (Table 2), which is consistent with the insular origin of the majority of extant kakapo. This last point also lends some support to the idea that, although kakapo have been through a demographic bottleneck, the extant population may not yet have passed through a genetic bottleneck (*cf.* black robin *Petroica traversi*; Table 2) (Graeme Elliott *in litt.*). Indeed, as Elliott points out, all the extant breeding adult kakapo are still survivors of a larger population. Whether this is the case will be difficult to resolve because very few DNA samples exist for previous generations of the Stewart Island kakapo population (i.e., only three museum

samples potentially exist from Stewart Island: Williams 1960; Dawson 1962).

Minisatellite DNA fingerprinting has also shown that the last Fiordland kakapo, Richard Henry, represents a separate lineage from the Stewart Island birds (Miller *et al.* 2003): a point supported by the fact that the only variation at the kakapo-specific microsatellite loci was found in Richard Henry (see above; B. Robertson unpubl. data; Miller *et al.* 2003). As such, Richard Henry is important to the ongoing genetic management of the kakapo as he alone provides the novel alleles necessary to boost the otherwise low genetic diversity of kakapo. Richard Henry is also the only source of novel variation with which managers could attempt to affect a "genetic rescue" of the kakapo (Ingvarsson 2001; but see Tallmon *et al.* 2004). Given that Richard Henry produced three offspring (2♀, 1♂) in the 1999 breeding season, a genetic rescue might already be in progress.

PATERNITY AND REPRODUCTIVE SUCCESS

Background

In many taxa, including birds, paternity is not a straightforward translation from observed social interactions and matings to paternity (Birkhead & Møller 1998). Both males and females tend to pursue alternative reproductive strategies (e.g., extra-pair paternity: Birkhead & Møller 1998; but see Robertson *et al.* 2001). Moreover, in birds, the ability to store sperm between successive matings further clouds which males are the genetic parents of offspring in the population (Birkhead 1998). Kakapo are no exception to this trend, hence genetics has been pivotal in assessing paternity in kakapo.

In kakapo, matings are typically well documented, but confusion still exists with regards to paternity. Female visitation of male track and bowls (Merton *et al.* 1984) now is remotely monitored by detecting individual female radio transmitter frequencies using a combined radio frequency scanner and logger (SNARKS: Eason *et al.* 2006). Also, kakapo leave distinctive feather clusters (mating sign) when they copulate (Powlesland *et al.* 1992, 2006; Eason *et al.* 2006). Despite this, paternity can be ambiguous because matings may take place away from the male's track and bowl (D. Eason unpubl. data). Also, females are known to mate with multiple males per clutch laid (Eason *et al.* 2006), which raises the possibility of mixed paternity of broods.

Paternity is easily resolved using genetic analysis and a number of molecular methodologies are available to assign genetic relationships, including microsatellite and minisatellite DNA fingerprinting (see section above). With knowledge of paternity also comes an understanding of the reproductive success of individual males, which in kakapo is expected to show high variance due to the species' lek mating system (Merton *et al.* 1984; Höglund & Alatalo 1995). High variance in reproductive success contributes to the loss of genetic variation, as noted above, because it leads to skewed lifetime production of offspring per individual, which in turn reduces the theoretical population size (effective population size, N_e) below that of the census population size, N (Frankham *et al.* 2002). The reduction in population size exacerbates the rate of loss of genetic variation as the rate of loss is now $1/2N_e$ per generation (Frankham *et al.* 2002). To stem the loss of genetic variation, managers could attempt to limit the variance in reproductive success by controlling which individuals obtain paternity in population. In kakapo, one way to alter an individual's reproductive success is to remove the individual from the breeding population (i.e., genetic management of kakapo).

The assignment of paternity has other important implications for the genetic management of kakapo. Understanding genetic relationships in the population also provides kakapo managers with the opportunity to develop a stud book and subsequently manage breeding to limit matings between close relatives. This is now especially pertinent because managers are planning on including artificial insemination among the strategies to increase kakapo productivity (DoC in press).

In the 1996-2005 kakapo recovery plan (Cresswell 1996) microsatellites markers were identified as the molecular marker of choice to examine kakapo paternity because managers wanted to determine paternity of all kakapo, including recently deceased kakapo (e.g., early death embryos and dead nestlings). Managers also wanted to identify individual kakapo via DNA from small tissue samples (i.e., feathers and fecal samples; G. Elliott pers. comm.). Unfortunately, insufficient allelic variability at microsatellite loci (see above) has made them largely uninformative for analysis of paternity, other than in cases where Richard Henry was a putative father. For example, the only allelic variation found in the extant kakapo (microsatellite locus *Sha*1: Robertson *et al.* 2000b) identified Richard Henry as the father of Sinbad and Kuia (B. Robertson unpubl. data; Miller *et al.* 2003). Subsequent paternity analyses have employed minisatellite DNA fingerprinting (Miller *et al.* 2003; B. Robertson & N. Gemmell unpubl. data).

Empirical findings

Minisatellite DNA fingerprinting has been informative in assigning the paternity of kakapo chicks (Miller *et al.* 2003). Of 13 chicks from eight clutches produced since 1991, paternity of 11 chicks could be assigned unequivocally. For the remaining two chicks (i.e., Morehu and Sirocco), examination of bandsharing between putative fathers and the chick allowed paternity to be assigned to two putative fathers in each case. Paternity was then assigned to one of these males using behavioural observations (Miller *et al.* 2003).

To remedy the limitations of minisatellite DNA fingerprinting in determining paternity of kakapo, paternity has been examined using the approach of

Table 3 The impact of variance in reproductive success and/or unequal sex ratios on the effective population size (N_e) of kakapo, based on breeding between 1992 and 1999 (Miller *et al.* 2003). For further details see Frankham *et al.* (2002).

	Males	Females
Adult numbers (N)	30	20
k*	3.25	2.17
V_k#	6.91	2.57
N_e due to variance in reproductive success (RS)	22.1†	18.0§
Overall N_e due to variance in RS & unequal sex ratios	39.7‡	

* k is the mean of the number of offspring contributed to the next generation. # V_k is the variance of the number of offspring contributed to the next generation. † $N_{em} = (N_m k\text{-}1)/[k\text{-}1+(V_k/k)]$, where N_m is the census number of adult males. § $N_{ef} = (N_f k\text{-}1)/[k\text{-}1+(V_k/k)]$, where N_f is the census number of adult females. ‡ $N_e = 4N_{ef}N_{em}/(N_{ef}+N_{em})$

Miller *et al.* (2003) and, when required, up to three other recently-identified variable molecular markers (B. Robertson & N. Gemmell unpubl. data). These markers include a microsatellite locus isolated from kaka (*Nestor meridionalis*) (i.e., *Nmed*17 contains three alleles in kakapo; Sainsbury *et al.* 2004; J. Sainsbury unpubl. data); the peptide binding region of exon 2 (a class II B gene) of the major histocompatability complex (MHC) region amplified using PCR primers designed for budgerigar (*Melopsittacus undulates*; Edwards *et al.* 1999); and three AFLP loci (amplified fragment length polymorphism: Vos & Kuiper 1998; Avise 2004) amplified using *Eco*RI and *Taq*I selective AFLP primers (i.e., two loci amplified with *Eco*RI (ACA) & *Taq*I (AGC); one locus amplified with *Eco*RI (AGC) & *Taq*I (AGC); see Vos & Kuiper 1998 for details; B. Robertson, H. Flint & N. Gemmell unpubl. data). Using this new approach, paternity of all 24 chicks fledged in the 2002 breeding season was assigned in each case to a single father and both ambiguous cases of paternity noted in Miller *et al.* (2003) were unequivocally assigned to a single father (B. Robertson & N. Gemmell unpubl. data).

As predicted from the species' lek mating system, paternity analysis has uncovered high variance in the reproductive success of male kakapo. Only four adult males of a possible 30 obtained paternity between 1991 and 1999 and one of these, Felix, fathered seven of the 13 chicks produced in this period (Miller *et al.* 2003). Using these findings, it is possible to assess the impact of skewed reproductive success and the combined impact of skewed reproductive success and unequal sex ratios on the effective population (N_e) of kakapo (Table 3). For example, the combined effect of the observed variance in reproductive success and the unequal sex ratio resulted in the effective population size of kakapo being only 79.3% that of the census population size (i.e., 50 adult kakapo in this period: Elliott *et al.* 2006).

Genetic management of the kakapo population could raise the N_e closer to N, but realistically only by manipulating male reproductive success. This is because males are not a limiting factor in kakapo productivity. High variance in male reproductive success could be reduced by excluding successful males (e.g., Felix) from the breeding population thereby allowing other males to reproduce. The only danger in this approach is if overall productivity is reduced as a result of the removal of a "hotshot" male from the lek; that is, if kakapo leks conform to the "hotshot" hypothesis (Höglund & Alatalo 1995).

FUTURE DIRECTIONS IN KAKAPO GENETICS

Genetics has clearly made important contributions to kakapo recovery and will continue to play an important role in the future. Advances in molecular methods and novel applications of existing methods (e.g., telomere ageing: see below) have the potential to continue to make genetics a valuable source of information for kakapo managers. Below I outline three new genetic studies of kakapo that could directly influence management strategies.

Genetic diversity and disease resistance

The molecular markers examined to date in kakapo (i.e., microsatellites and minisatellites markers) assess selectively-neutral variation in parts of the genome colloquially referred to as junk DNA (but see Hammock & Young 2005). Low genetic variation at such neutral markers might not have important fitness consequences but some genes play vital roles in how individuals interact with their environment. One group of fitness-related genes is the major histocompatibility complex (MHC), which codes for immune response in vertebrates (Edwards *et al.* 1998). Loss of variation from MHC genes can result in lower resistance to existing and/or novel pathogens (O'Brien & Evermann 1988; Reid *et al.* 2003).

The kakapo MHC is being examined using PCR primers designed for the peptide binding region of exon 2 (a class II B gene) of the budgerigar (*Melopsittacus undulates*; Edwards *et al.* 1999) (B. Robertson unpubl. data). This study is a first step in informing kakapo managers of the level of genetic variation present in kakapo MHC genes and will provide some very general indication of the possible immune system response of kakapo to potential future novel pathogens, such as the spread of avian malaria in New Zealand (D. Tompkins unpubl. data).

Molecular ageing

Knowledge of age structure is an important piece of information in species recovery planning, especially because age-specific fecundity and mortality can impact on population growth potential (Albon *et al*. 2000; Coulson *et al*. 2001). Logically, different management actions would be required to recover a small population of long-lived individuals whose breeding population is on the verge of reproductive senescence compared with a population just reaching the peak of fecundity. For example, hormone therapy to extend the breeding life of females might be prioritized as a management option in a senescing population (e.g., Objective 2.7 of the 1996-2005 kakapo recovery plan: Cresswell 1996).

In natural populations of long-lived species, age is rarely known because it either requires costly extensive longitudinal studies or destructive sampling (e.g., growth rings of otiliths in fish). The crisis nature of species recovery often rules out long-term studies to determine age and obviously destructive sampling is not an option. Recently, it has been shown that telomeres (i.e., short tandem-repeated sequences of DNA at ends of eukaryotic chromosomes: Nakagawa *et al*. 2004) normally shorten with age (Haussmann & Vleck 2002; Haussmann *et al*. 2003; Vleck *et al*. 2003). Telomere length appears to correlate with lifespan (Haussmann *et al*. 2003; Vleck *et al*. 2003), hence potentially providing a straightforward method to determine age (Nakagawa *et al*. 2004).

For kakapo, breeding lifespan is largely unknown and few reproductively active individuals are of known age (Elliott *et al*. 2001; Eason *et al*. 2006; Elliott 2006): 16% of the 49 extant adult kakapo are of known age (i.e., 4 of 21 ♀♀; 4 of 28 ♂♂). Anecdotal evidence suggests that older males can produce viable sperm at a minimum age of 29: Richard Henry fathered offspring in 1999, but he could be considerably older (up to a minimum age of 46 years) because female kakapo or signs of breeding were not seen in the Milford Sound area from the first Wildlife Service expedition there in 1958 up to his capture in 1975 (Butler 1989). For females, available evidence suggests that individuals can breed at a minimum age of 33 years: Alice breed in 2005 but produced an infertile clutch of three eggs (D. Eason unpubl. data).

With most unknown age kakapo having a minimum age of greater than 20 years (22.6 ± *sd* 5.1 years, $n = 41$), the possibility exists that a large proportion of the reproductively-active individuals are at, or past, the peak of fecundity and possibly nearing reproductive senescence. This would have important implications for kakapo management. Research currently being undertaken is examining the relationship between telomere length and lifespan in kakapo (T. Horn, B. Robertson & N. Gemmell unpubl. data). This genetic approach to ageing kakapo may provide much needed information on population age structure with which to model population trends and guide future management strategies (Elliott 2006).

Genetic similarity and hatching success

Poor hatching success in birds has been suggested as a fitness consequence of high genetic similarity (Spottiswoode & Møller 2003) and also correlates with low levels of genetic diversity as inferred from demographic bottleneck severity (Briskie & Mackintosh 2004). For birds, about 10% of eggs fail to hatch (Koenig 1982), but in kakapo this value is about 58% (83 of 146 eggs laid between 1981 and 2002: Elliott *et al*. 2006). Given the apparent relationship between genetic variation and hatching success, low hatching success in kakapo may be a consequence of the low genetic diversity in the species: a point that has only recently been given serious consideration possibly due to a paradigm shift in NZ conservation (see Jamieson *et al*. in press).

Research currently being undertaken on kakapo aims to examine the relationship between the genetic similarity of parents and the hatching success of eggs. Using minisatellite DNA bandsharing coefficients (see above) as an indication of relatedness between mated individuals (e.g., Brock & White 1992) and information on nesting success of past breeding attempts (D. Eason unpubl data), this study will examine if higher levels of genetic similarity between breeding individuals result in lower hatching success. Information on the genetic similarity of individuals would also allow the investigation of genetic relationships among the founding members of the extant kakapo population (Haig *et al*. 1994), the majority of which are of unknown genealogy (D. Eason unpubl. data). This information could be used by kakapo managers in an attempt to minimize breeding between closely related individuals and, in doing so, possibly improve the hatching success of kakapo and hence the rate of species recovery.

CONCLUSION

Despite the general absence of genetic variation among the surviving kakapo, genetic approaches have provided a wealth of information to kakapo managers over the last 10 years, much of which has had a direct impact on population management. For example, genetic research identified the impact of supplementary feeding on brood sex ratios, thereby allowing kakapo managers to redress the male bias in productivity (Clout *et al*. 2002; Robertson *et al*. in press).

As such, genetics should remain an important component of kakapo recovery but not at the expense of productivity. Genetic information alone will not be the panacea of kakapo recovery, but it should be integrated with other management goals to ensure that future generations of kakapo retain maximal genetic variation.

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