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Conservation genetic management of a critically endangered New Zealand endemic bird: minimizing inbreeding in the Black Stilt *Himantopus novaezelandiae*

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For threatened species with small captive populations, it is advisable to incorporate conservation management strategies that minimize inbreeding in an effort to avoid inbreeding depression. Using multilocus microsatellite genotype data, we found a significant negative relationship between genetic relatedness (inbreeding) and reproductive success (fitness) in a captive population of the critically endangered Black Stilt or Kākī *Himantopus novaezelandiae*. In an effort to avoid inbreeding depression in this iconic New Zealand endemic, we recommend re-pairing closely related captive birds with less related individuals and pairing new captive birds with distantly related individuals.

Keywords: inbreeding depression, microsatellites, genetic relatedness, reproductive success, Kākī.

To ensure the long-term survival of threatened species, the genetic factors that increase extinction risk, such as inbreeding depression, must be addressed (Reed & Frankham 2003, Frankham 2005, Jamieson *et al.* 2006). Inbreeding depression, is a decrease in fitness caused primarily by the expression of deleterious recessive alleles in individuals that are highly homozygous as a consequence of inbreeding (i.e. mating amongst close relatives; Keller & Waller 2002). Inbreeding can rapidly accumulate in small populations as mating amongst close relatives is often unavoidable. Consequently, inbreeding depression is likely to represent a significant extinction risk for declining populations of many species worldwide (Spielman *et al.* 2004). This risk is further exacerbated when conservation management of the species includes a captive breeding population, because captive populations are often drawn from a smaller pool of wild stock. Thus, for threatened species with small captive populations, it is particularly advisable to incorporate strategies that minimize inbreeding in

an effort to avoid inbreeding depression (Hedrick & Kalinoswki 2000).

Inbreeding depression has been documented in many wild populations (for a review see Keller & Waller 2002) and in birds often manifests itself as a decline in hatching success or the survival of young chicks (Keller & Waller 2002, Briskie & Mackintosh 2004, Heber & Briskie 2010). However, the decrease in fitness due to inbreeding can occur and accumulate across all life stages, as recently documented in Great Tits *Parus major* (reduced hatching success, fledging success and recruitment; Szulkin *et al.* 2007) and Takahe *Porphyrio hochstetteri* (accumulated decrease in fitness across all life stages equivalent to an 88% reduction in recruitment of second-generation offspring; Grueber *et al.* 2010). To minimize inbreeding depression in the most efficient and effective manner, managers must first: (1) calculate an appropriate measure of inbreeding, and (2) test that a significant negative relationship between inbreeding and fitness exists.

When managing captive pairs, the most appropriate measure of inbreeding is the relatedness of prospective parents. Relatedness can be accurately

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estimated directly from pedigree data (Frankham *et al.* 2002, Pemberton 2004), but because confirmation of parentage is extremely difficult in most natural populations, these data are not available for many species. Instead, relatedness calculated from multilocus microsatellite genotypes is often used as a surrogate (Pemberton 2004, 2008). In this study, we use multilocus microsatellite data to examine the relationship between genetic relatedness (inbreeding) and reproductive success (fitness) in a captive population of the critically endangered Black Stilt or Kakī *Himantopus novaeseelandiae*, and use these data to make explicit recommendations for the Kakī captive-breeding programme.

One of the rarest and most threatened birds in the world, Kakī are endemic to the braided rivers of New Zealand (BirdLife International 2010). Predation by introduced mammals and widespread habitat loss and degradation were key factors in the species' decline to approximately 23 adults in 1981 (Maloney & Murray 2001, Steeves *et al.* 2010). From that year, intensive conservation management activities began in the Upper Waitaki Basin, including a captive-breeding and captive-rearing programme: eggs collected from captive and wild pairs are incubated in captivity, chicks are then brooded and reared in captivity, and juveniles or subadults are subsequently released to the wild, or are held in captivity to form the basis of a small (six pairs) captive flock (Maloney & Murray 2001). Although recruitment is low (van Heezik *et al.* 2009), Kakī numbers are increasing: in 2010 there were 98 birds in the adult population (85 wild and 13 captive). With such low numbers, Kakī remain at risk of extinction by deterministic and genetic factors (Maloney & Murray 2001, Steeves *et al.* 2010). Thus, a multi-faceted conservation management approach is required to ensure the long-term survival of this iconic bird.

Kakī in the captive-breeding programme are currently paired from small groups of chicks held back from release and raised to breeding age (2 years; Maloney & Murray 2001) in captivity. Kakī are strongly territorial, and pairs in captivity will not tolerate other birds in their vicinity (Maloney & Murray 2001). Establishing new pairs can therefore be very challenging. New pairs are formed by flock-mating: three to five unmated birds are placed in the same aviary space for a few hours to allow them to form potential pair bonds. The resulting pair bonds are often unpredictable, and can be with any of the potential mates. Thus,

there is a large degree of uncertainty about which birds will pair together. Once established, pairs form life-long bonds (Maloney & Murray 2001).

Some captive pairs can be exceptionally productive and lay up to four fertile eggs in each of four clutches per year, whereas others may lay few, if any, eggs and eggs may frequently fail to hatch (van Heezik *et al.* 2005). One potential explanation for the observed pattern is that less productive pairs are closely related (i.e. their offspring are inbred; Keller & Waller 2002). Given the substantial effort required to establish and sustain breeding pairs and their offspring in captivity, and the subsequent low levels of production of some pairs, an examination of the relationship between inbreeding and fitness in the captive Kakī population is timely.

METHODS

We examined genetic relatedness and reproductive success for captive pairs from 1998 to 2009 held in facilities at the Department of Conservation in Twizel and at Peacock Springs in Christchurch. Data for captive pairs prior to 1998 were excluded because these data pre-dated the standard introduction of an iodine supplement in the captive diet, which dramatically improved breeding success of captive pairs (Sancha *et al.* 2004). We further excluded data for now deceased captive pairs for which tissue samples were unavailable (nine individuals contributing to seven captive pairs) and where genotype data were missing (two individuals contributing to one captive pair).

We collected genotypes at eight microsatellite loci (BS2, BS9, BS12, BS13, BS21, BS27, BS40 and BSdi7; Steeves *et al.* 2008) for 21 individuals from 14 pairs: data for 14 individuals came from a previous study (Steeves *et al.* 2010) and for the remaining seven individuals we followed the methods detailed in Steeves *et al.* (2010). Standard estimates of genetic diversity for these eight microsatellite loci including tests for significant deviations from Hardy–Weinberg equilibrium and linkage disequilibrium can be found elsewhere (Steeves *et al.* 2008, but see Supporting Information, Table S1, for raw data and Table 1 for allele frequency, observed heterozygosity and expected heterozygosity data for this study).

Given the complex history of extensive human-induced bidirectional hybridization between Kakī and Pied Stilts (Poaka) *Himantopus himantopus leucocephalus* (for details see Steeves *et al.* 2010),

Table 1. Allele frequencies and observed (H_O) and expected (H_E) heterozygosity at eight microsatellite loci in Kaki.

Locus (n)	Allele size (bp)	Allele frequency	H_O	H_E
BS2 (21)	132	0.310	0.524	0.666
	136	0.357		
	140	0.333		
BS9 (21)	119	0.143	0.619	0.668
	127	0.405		
	131	0.381		
	132	0.024		
	139	0.048		
BS12 (21)	245	0.857	0.286	0.251
	249	0.119		
	253	0.024		
BS13 (21)	175	0.524	0.667	0.499
	187	0.476		
BS21 (21)	229	0.452	0.762	0.686
	233	0.262		
	237	0.143		
	241	0.143		
BS27 (21)	200	0.690	0.476	0.473
	204	0.119		
	208	0.190		
BS40 (21)	132	0.786	0.143	0.353
	140	0.167		
	145	0.048		
Bsd17 (19)	190	0.026	0.474	0.573
	208	0.079		
	210	0.579		
	212	0.026		
	214	0.289		

we confirmed that all individuals analysed in this study were genetically Kaki using the Bayesian admixture methods outlined in Steeves *et al.* (2010) prior to all downstream analyses. Briefly, we used the Bayesian clustering algorithm implemented in Structure 2.3 (Pritchard *et al.* 2000, Hubisz *et al.* 2009) to estimate an assignment probability to 'Kaki' and 'Poaka' clusters for each sample. Individuals were classified as genetically Kaki when the assignment probability to the 'Kaki' cluster was ≥ 0.95 .

We used GENALEX 6.4 (Peakall & Smouse 2006) to calculate Queller and Goodnight's (1989) genetic estimate of pairwise relatedness, r , for each captive pair. Despite efforts to collect familial data since active conservation management began in 1981, we were unable to calculate a meaningful estimate of relatedness for each captive pair directly from the Kaki pedigree data because it is both shallow (i.e. includes kin relationships from one to four generations) and incomplete

(i.e. contains offspring from unbanded birds, particularly among the founder population).

To visualize the relationship between genetic relatedness and reproductive success, we drew a scatter plot and represented each captive pair by circles whose size was relative to the total number of eggs laid. We used hatching success (measured as the proportion of eggs laid per pair that successfully hatched) as a measure of reproductive success. Because eggs were artificially incubated in standard conditions, hatching failure is directly related to intrinsic rather than extrinsic causes of failure (van Heezik *et al.* 2005).

To examine statistically the relationship between genetic relatedness and reproductive success, we used generalized linear mixed effects models (GLMMs, Bolker *et al.* 2009), which allow for the inclusion of random effects and non-normal errors. In GLMMs we considered egg hatching as the binomial response variable. In addition to genetic relatedness (our primary fixed effect of interest), we also explored the possibility that pair experience may contribute to reproductive success and included as additional fixed effects the independent ages of each partner in the pair and the cumulative number of clutches the pair had laid together. To account for the non-independence of eggs within a clutch, and eggs originating from given parents, we included clutch and parent identity as random effects in the models. We constructed a set of models including all combinations of fixed effects; each model included all random effects. We fitted GLMMs with the lme4 package (Bates & Maechler 2010) in R 2.8.1 (available at <http://www.R-project.org>) with maximum-likelihood approximation. To identify the model with the best fit to the data, we used a likelihood ratio test to compare models including different combinations of fixed effects with a null model including only the intercept and random effects (Table 2). Our final model was fitted with the Laplace approximation and contained genetic relatedness as the fixed effect, and clutch and parent identity as random effects.

A significant negative relationship between genetic relatedness and reproductive success may be due to either local effects (i.e. due to linkage disequilibrium between marker and fitness loci) or general effects (i.e. due to inbreeding) (Grueber *et al.* 2008, Hansson & Westerberg 2008). To test for local effects, we repeated the GLMM analyses described above for the seven remaining loci after

Table 2. Alternative models are listed by their main fixed effects. Each model listed also included an intercept and random effects of clutch and parent identities. Models were compared using a maximum-likelihood approximation to a null model that included only the intercept and random effects. Resulting Akaike Information Criterion scores are shown (AIC, equal to $2 \times \log\text{-likelihood} + 2 \times (\text{number of model parameters} + 1)$), as well as the difference in AIC score with the best-fitting model of the set (ΔAIC).

Model	Log-likelihood	AIC	ΔAIC
Relatedness + clutch + male age + female age*	-252.06	520.12	4.98
Relatedness + clutch + female age	-252.39	518.78	3.64
Relatedness + clutch + male age	-252.07	518.13	2.99
Relatedness + male age + female age	-252.08	518.15	3.01
Clutch + male age + female age	-255.44	524.89	9.75
Relatedness + clutch	-252.40	516.80	1.66
Relatedness	-252.57	515.14	0.00

*Relatedness, genetic relatedness; clutch, cumulative number of clutches pair laid together; male and female age, age of parent at time of laying.

each locus was removed sequentially. Physical linkage among any of the eight microsatellite loci used in this study appears unlikely (Steeves *et al.* 2008), so removing one locus at a time should indicate whether linkage between marker and fitness loci is responsible for any observed relationship between genetic relatedness and reproductive success. To test for general effects, we used Excel 2007 to calculate a distribution of heterozygosity–heterozygosity correlations. We did this from 1000 randomizations comparing individual heterozygosity at four randomly chosen loci with individual heterozygosity at the remaining four loci, across all 21 individuals.

RESULTS

Prior to conducting all downstream analyses, we confirmed that all birds in this study were genetically Kakī: assignment probabilities ranged from 0.988 to 0.996 (i.e. assignment probabilities were ≥ 0.95 ; for details see Steeves *et al.* 2010). We examined reproductive success for 131 clutches (440 eggs from 21 birds in 14 pairs) and found that reproductive success decreased as genetic relatedness increased (Fig. 1) and that this negative relationship was significant (Tables 2 and 3).

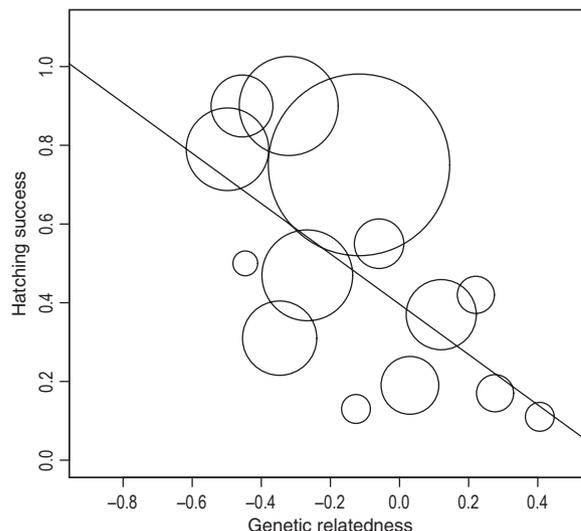


Figure 1. Relationship between genetic relatedness and hatching success among pairs in the Kakī captive breeding programme from 1998 to 2009. Pairs are represented by circles whose size is relative to the total number of eggs laid by each pair. The solid black line represents the trend.

We removed each locus one at a time and found that the GLMM analyses remained significant in all cases except removal of BSdi7, which resulted in a marginally non-significant ($P = 0.09$) negative relationship between genetic relatedness and reproductive success (Table 3). This result is likely to be due to the fact that BSdi7 has a number of rare alleles (three of five), which are known to heavily influence calculations of genetic relatedness (Roberts *et al.* 2006). Thus, by removing BSdi7 we effectively removed the locus with the most power to differentiate amongst pairs. Despite this reduction in power, genetic relatedness values calculated from seven loci (minus BSdi7) are significantly correlated with those calculated from all eight loci ($r = 0.87$, $P < 0.0001$). Thus, the significant negative relationship between genetic relatedness and reproductive success observed for all eight loci in the captive Kakī population is unlikely to be driven by a strong association between marker and fitness loci. There was a small but positive mean heterozygosity–heterozygosity correlation (mean $r = 0.159 \pm 0.003$ sd, 1000 randomizations), which suggests that diversity at the eight loci used in this study is likely to be indicative of genome-wide diversity, and thus estimates of relatedness calculated from these multilocus microsatellite genotypes are a reasonable surrogate for relatedness calculated from a Kakī pedigree.

Table 3. Coefficients of a binomial generalized linear mixed effect model with hatching success as a binary response, genetic relatedness as a fixed effect, and clutch and parent identities as random effects.

Model	Fixed effects	Estimate (se)	Z value	P value
All loci included	Genetic relatedness	-2.89 (0.86)	-3.35	0.0008
	Intercept	-0.21 (0.39)	-0.53	0.59
Locus BS2 removed	Genetic relatedness	-2.51 (1.04)	-2.41	0.02
	Intercept	-0.12 (0.40)	-0.29	0.77
Locus BS9 removed	Genetic relatedness	-3.05 (0.77)	-3.96	0.00008
	Intercept	-0.13 (0.32)	-0.40	0.69
Locus BS12 removed	Genetic relatedness	-2.96 (0.82)	-3.60	0.0003
	Intercept	-0.19 (0.37)	-0.52	0.60
Locus BS13 removed	Genetic relatedness	-2.95 (0.88)	-3.36	0.0008
	Intercept	-0.31 (0.41)	-0.75	0.45
Locus BS21 removed	Genetic relatedness	-2.42 (0.69)	-3.49	0.0005
	Intercept	-0.23 (0.41)	-0.57	0.57
Locus BS27 removed	Genetic relatedness	-2.34 (0.78)	-3.00	0.003
	Intercept	-0.14 (0.45)	-0.32	0.75
Locus BS40 removed	Genetic relatedness	-2.42 (0.82)	-2.94	0.003
	Intercept	-0.08 (0.45)	-0.17	0.87
Locus BSdi7 removed	Genetic relatedness	-1.42 (0.83)	-1.72	0.09
	Intercept	0.09 (0.46)	0.20	0.85

DISCUSSION

These combined data indicate that, despite genotyping a relatively small number of marker loci for a limited number of individuals, our estimates of genetic relatedness are an appropriate measure of inbreeding in Kakī and that a significant negative relationship between inbreeding and fitness does indeed exist in the captive Kakī population. Thus, avoiding inbreeding depression is warranted by conservation managers. We recommend minimizing inbreeding by (1) re-pairing highly related captive birds with less related individuals, and (2) pairing new captive birds with individuals that are as distantly related as possible. In addition to increasing the productivity of captive pairs, implementing these recommendations will probably decrease the extinction risk of Kakī by maximizing individual heterozygosity (Frankham 2005).

Prior to pair formation, genetic relatedness will need to be estimated for all prospective parents. Although relatedness calculated from extensive pedigrees is more accurate than that calculated from microsatellite markers (Pemberton 2004, 2008), microsatellite markers are still useful in assessing close relationships among captive individuals where pedigree data are incomplete (Ivy *et al.* 2009, but see Grueber *et al.* 2011). However, given the small size of the Kakī population, and because almost all Kakī are now reared in captivity, the captive Kakī pedigree will eventually contain

all but the deepest relationships among living adults. Balloux *et al.* (2004) suggest that a pedigree of only three to five generations is sufficient for reasonably accurate estimates of inbreeding (and therefore relatedness of parents), and the Kakī pedigree is approaching that level of depth for many individuals. However, until that level of information is available for all adults, our data indicate that microsatellite genotypes can be used to assess relatedness, fill in gaps in the pedigree and ensure no closely related individuals are allowed to pair. As part of a comprehensive conservation management approach, future efforts to minimize inbreeding in the captive Kakī population will play an important role in the continued recovery of a critically endangered New Zealand endemic.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Genotypes of 21 Kākī individuals at eight microsatellite loci.

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