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Pedigree validation using genetic markers in an intensively-managed taonga species, the critically endangered kakī (*Himantopus novaezelandiae*)

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Abstract: Many species recovery programmes use pedigrees to understand the genetic ancestry of individuals to inform conservation management. However, incorrect parentage assignment may limit the accuracy of these pedigrees and subsequent management decisions. This is especially relevant for pedigrees that include wild individuals, where misassignment may not only be attributed to human error, but also promiscuity (i.e. extra-pair parentage) or egg-dumping (i.e. brood parasitism). Here, we evaluate pedigree accuracy in the socially monogamous and critically endangered kakī (black stilt, *Himantopus novaezelandiae*) using microsatellite allele-exclusion analyses for 56 wild family groups across three breeding seasons (2014–2016, $n = 340$). We identified 16 offspring where parentage was incorrectly assigned, representing 5.9% of all offspring. Of the 16 misassigned offspring, three can be attributed to non-kakī brood parasitism, one can be assigned to human error, but others cannot be readily distinguished between non-monogamous mating behaviours and human error. In the short term, we advise the continued use of microsatellites to identify misassigned offspring in the kakī pedigree, and to verify non-kakī brood parasitism. We also recommend the Department of Conservation's Kakī Recovery Programme further evaluate the implications of pedigree error to the management of this critically endangered taonga species.

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INTRODUCTION

For threatened species that have experienced significant and sustained population decline, genetic management can be paramount to enhance recovery (Grueber *et al.* 2019). Pedigrees, or genealogical records amongst individuals in a population, are an invaluable tool for genetic management of highly threatened populations. Pedigrees allow conservation practitioners to track diversity over time and strategically pair or translocate individuals to minimise inbreeding and maximise genome-wide diversity (Farquharson *et al*. 2017; Galla *et al*. 2020). While pedigrees are commonly used to manage captive populations (i.e. *ex situ*; Ballou *et al*. 2010), there are rare instances where they are maintained for wild populations (i.e. *in situ*; Pemberton 2008). Historically, pedigrees of wild populations have relied on behavioural data and field observations of social pairings to confirm parentage (Keller & Waller 2002), but the accuracy of these wild pedigrees can be compromised when parents are incorrectly assigned to putative offspring.

Incorrect parentage assignment for pedigrees can be attributed to either human error or unexpected and undetected mating behaviour. Human error can include misidentification of individuals in the field (e.g. misread coloured leg bands, or dropped leg bands in birds; Milligan *et al*. 2003) or transcription errors (Oliehoek & Bijma 2009). For example, a recent molecular study in Attwater's prairie-chicken (*Tympanuchus cupido attwateri*) found a 4.1% pedigree error rate attributable to human error in the pedigree of captive individuals (Hammerly *et al*. 2016). In addition to human error, undetected and non-monogamous mating behaviour can also affect the pedigree of wild individuals, as breeding pairs are not confined in separate enclosures. Numerous genetic studies in birds show that social mates may not be the genetic parents of their putative offspring due to brood parasitism or extra-pair parentage (Firth *et al*. 2015). Avian brood parasitism is defined by laying one's eggs in the nest of another individual and providing no additional parental investment (Davies 2000). Using this reproductive strategy, the donor parents outsource the cost of rearing their offspring to the recipient parents. Some bird species, such as the cuckoo finch (*Anomalospiza imberbis*), are obligate brood parasites, reproducing only through laying their eggs in the nests of other species (Sorenson & Payne 2002). Others, such as some species of stilts (*Himantopus* spp.), participate in facultative brood parasitism by laying eggs in the nests of others while also tending their own nests (Yom-Tov 1980; Overbeek *et al*. 2017). Extra-pair parentage occurs when one, or both individuals, mate with another outside of a socially monogamous pairing (Petrie *&* Kempenaers 1998), resulting in a discrepancy between one parent of the nest and their putative offspring. This can include extra-pair paternity (Westneat *et al*. 2003) where the social father is not the genetic father of offspring, and quasi-parasitism (Petrželková *et al*. 2015) where the social mother is not the genetic parent of offspring. Extra-pair parentage is common in socially monogamous birds such as the Eurasian magpie (*Pica pica*; Birkhead & Biggins 1987; Westneat *et al*. 1990; Davies 2000) and the reed bunting (*Emberiza schoeniclus*), where extra-pair paternity rates run as high as 55% (Griffith *et al*. 2002). In Aotearoa New Zealand, the tui (*Prosthemadera novaeseelandiae*) is an excellent example of extra pair paternity, with extra pair offspring accounting for 57% of all young (Wells *et al*. 2015). With potential for promiscuous breeding behaviour in the wild, it is inadvisable to ascertain parentage for wild pedigrees based on field observations alone.

One species whose management benefits from a pedigree of captive and wild individuals is the critically endangered kakī, or black stilt (*Himantopus novaezelandiae*, Figure 1). Kakī were previously found on both the North and South Islands of Aotearoa, but experienced significant decline in the 19th and 20th centuries through the impact of non-native mammalian predators and habitat loss (Reed & Murray 1993). As of April 2020, the contemporary breeding population of kakī consists of 169 wild adults that are largely confined to Te Manahuna / the Mackenzie Basin (Department of Conservation, *pers. comm*.). The Department of Conservation (DOC) initiated the Kakī Recovery Programme in the early 1980's to enhance recovery efforts for the species; management practices to date include predator control, intensive monitoring of wild birds, management of hybridisation with poaka / pied stilts (*H. himantopus leucocephalus*), and a conservation breeding and rearing programme (Maloney & Murray 2001). In an effort to reduce predation of eggs and young chicks in the wild, eggs are collected from wild nests, artificially incubated, and captive reared by hand before individuals are banded and released back into the wild as juveniles or sub-adults (van Heezik *et al.* 2005). For captive breeding, kakī are strategically paired in captivity (2–7 pairs) to minimise inbreeding and maximise diversity (Galla *et al.* 2020). A recent study investigating relatedness estimates in captive and wild kakī showed that pedigree- and genomic-based relatedness coefficients and subsequent pairing recommendations correlate significantly with one another (Galla *et al*. 2020). While this strong correlation provides confidence in the kakī pedigree, a small number of individuals showed unexpected discrepancies between pedigree- and genomic-based relatedness. Thus, a rigorous investigation of the accuracy of the pedigree, specifically for offspring of wild pairs, is warranted.

Figure 1. An adult kakī in the Tasman Valley of Te Manahuna (Photograph: Liz Brown).

The kakī pedigree is generally assumed to be accurate for wild individuals, as kakī are identifiable through unique coloured leg bands, intensively monitored, and socially monogamous. However, a 2017 study using microsatellite markers and phenotypic data revealed the first evidence for brood parasitism in kakī from 'non-kakī' stilts (i.e. poaka, or kakī-poaka hybrids; Overbeek *et al*. 2017). These birds were easily identified as being atypical, as they displayed pale plumage compared to other kakī of the same age. In recent breeding seasons, the Kakī Recovery Programme has also kept lists of uncertainty in the pedigree that may be the result of human error. For example, in 2018, two chicks from two different clutches were recorded having dropped leg bands overnight in the same brooder box (Department of Conservation, *pers. comm.*). To verify which chicks belonged to putative wild parents, microsatellites were amplified across unknown individuals, their siblings, and possible parents to assign them to their putative parent group.

While these practices can be used to identify pedigree discrepancies that are the result of known human error and non-kakī brood parasitism, the programme has not examined whether all wild offspring are correctly assigned to their putative parents. In this study, we examine the accuracy of the pedigree of wild kakī over three breeding seasons (2014–2016) using eight microsatellite markers and allele-exclusion analyses to identify Mendelian irregularities between putative parents and offspring. While these eight microsatellite markers cannot rule out false negatives (i.e. birds that appear to be the offspring of social — but not genetic — parents, as a result of shared common alleles), they do provide an opportunity to exclude putative parentage, which can reveal minimal pedigree error rates and inform best practice for managing the kakī pedigree moving forward.

MATERIALS AND METHODS

Genetic material sourcing and sampling

Animal ethics approval has been granted by DOC (permit number AEC 283). Since 1998, DOC has collected blood feathers from all juvenile kakī that have passed through the captive rearing and breeding programmes as a part of routine health checks. These feathers have been maintained in a -20°C freezer at the University of Canterbury since collection, and were used for this study. Samples chosen for analysis include all wild offspring from the 2014 (n = 20 families, 105 individuals), 2015 (n = 15 families, 56 individuals), and 2016 (n = 21 families, 112 individuals) breeding seasons that survived to banding age (25–35 days old) and their putative parents, as listed in the kakī pedigree (Galla *et al*. 2020). We only included offspring that survived to at least banding age in these analyses, as feather collections have traditionally included these individuals.

DNA extraction and microsatellite genotyping

Feather tips were placed into Eppendorf tubes using sterilized forceps and scissors. Initially, DNA was extracted using the Invitrogen™ PureLinkTM Genomic DNA Mini Kit (Thermo Fisher Scientific) following manufacturer instructions. However, a chelex method was found to be more efficient and produced equal or higher concentrations of DNA for kakī, and was used to extract the remaining samples in this study. Briefly, feather tips were suspended in 200 μL of a 5% Bio-Rad Chelex-100® chelating resin solution in PCR grade water with 20 μL of 20 mg/mL proteinase K. This solution was incubated at 56°C for 12 hours. For elution, the supernatant (~200 μL) was combined with 50 μL of TE buffer. Extraction success was verified using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific).

Eight microsatellite loci (BS2, BS9, BS12, BS13, BS21, BS27, BS40, BSdi7) originally described by Steeves *et al*. (2008) for use in *Himantopus* spp. were used in this study. Null alleles were not reported for these loci when they were originally described and none have been detected in the 12 years they have been in use. Seven of the eight loci used in this study are tetra-mers, which means that stutter patterns are readily resolved. The remaining locus (di7) is a di-mer; while the stutter patterns for this locus are more complex, they are also well-characterised. PCR amplifications for these loci were performed as described in Steeves *et al*. (2008). To verify successful PCR amplification, a subset of PCR products and negative controls were run on a 1.4% agarose gel

stained with Invitrogen SYBR® Safe Gel Stain at 90V for 45 minutes. For genotyping, 0.5 μL of PCR products were added to 0.3 μL of GeneScanTM 500 LIZ® size standard (Applied Biosystems) and 11.7 μL of formamide. Samples were run on an ABI 3130xl Genetic Analyzer (Applied Biosystems) and allele sizes were scored by eye using GENEMARKER v. 2.4 (SoftGenetics, State College PA, USA).

In instances of Mendelian mismatch (see below), mismatching parents and offspring were re-extracted and genotyped if extra feather samples for individuals were available. A genotyping error rate was calculated by dividing the number of corrected alleles by those that were available for comparison. The programme GENALEX v. 6.5 (Peakall & Smouse 2006; Smous & Peakall 2012) was used to calculate allele size, allele frequency, observed heterozygosity (H_O), and expected heterozygosity (H_E) at each microsatellite locus. Tests for linkage disequilibrium and deviations from Hardy-Weinberg equilibrium in kakī can be found elsewhere (Steeves *et al*. 2008; 2010).

Allele-exclusion analyses

Allele calls for offspring were checked against putative parents using allele-exclusion, a common method for examining parentage in both natural and experimental populations (Zhang *et al.* 1994; Maudet *et al.* 2002; Manel *et al.* 2005). This approach identifies mismatched putative parents and offspring through irregularities in Mendelian inheritance (Vandeputte *et al.* 2006). Mismatches were counted only when putative parents and offspring did not match at >1 allele, to account for potential random mutations (Ellegren 2000). All mismatched offspring were checked across field notes from the Kakī Recovery Programme, to consider whether atypical behaviour (e.g. abnormal nesting behaviour) or human error (e.g. note taking errors) could add context to mismatches. To test whether mismatched offspring were assigned as kakī or non-kakī, we implemented the Bayesian clustering algorithm in STRUCTURE v. 2.3.4 (Pritchard *et al*. 2000, as per Steeves *et al*. 2010) for all mismatched offspring to estimate assignment to kakī or non-kakī clusters. If assignment probabilities were <95% to the kakī cluster, offspring were identified as non-kakī and a 291bp fragment of the mitochondrial cytochrome *b* gene was sequenced as per Steeves *et al*. (2010) to verify the maternal haplotypes for these individuals.

RESULTS

For each of the 340 individuals sampled (56 family groups across the 2014–2016 breeding seasons), genotypes were obtained for at least seven of the eight microsatellite loci (data available at https://github.com/sgalla32/Kaki_Microsatellites). There

Table 1. Descriptive statistics for microsatellites used to validate the kakī pedigree, including allele size (base pairs), allele frequency, observed heterozygosity (H_O), and expected heterozygosity (H_E).

Locus	Allele Size	Allele Frequency	H_O	H_E
2	121	0.001	0.616	0.647
	125	0.001		
	132	0.231		
	136	0.438		
	140	0.329		
9	115	0.003	0.665	0.632
	119	0.128		
	127	0.409		
	131	0.428		
	139	0.032		
12	245	0.821	0.327	0.308
	249	0.119		
	253	0.054		
	257	0.001		
	267	0.003		
	288	0.001		
13	175	0.536	0.491	0.502
	187	0.460		
	195	0.004		
21	229	0.335	0.796	0.732
	233	0.167		
	237	0.294		
	241	0.205		
27	188	0.001	0.534	0.465
	192	0.001		
	200	0.700		
	204	0.171		
	208	0.126		
40	132	0.698	0.451	0.448
	140	0.249		
	145	0.052		
	150	0.001		
di7	190	0.033	0.609	0.591
	192	0.001		
	194	0.001		
	208	0.119		
	210	0.558		
	214	0.287		

was an observed range of 3–6 alleles per locus, with average observed heterozygosity (H_O = 0.56 ± 0.14) being slightly higher than expected heterozygosity (H_E = 0.54 ± 0.14; Table 1). Of the 52 individuals that were re-extracted and genotyped, 4.66% of 751 alleles were corrected.

Across the 56 family groups studied, nine had offspring with alleles that could not be attributed to one or both of their putative parents (n = 16 offspring, or 5.9% of offspring studied; Figure 2). In the 2014 breeding season, three family groups showed Mendelian mismatches between putative parents and offspring, including family groups with DOC identifiers 14/08, 14/09, and 14/13. The offspring from family group 14/08 were collected in two clutches from the wild, and all surviving offspring from both clutches have alleles at three loci that do not correspond with putative parents. While some of these mismatched alleles (i.e. loci 2 and 9) cannot be attributed to the mother, other mismatched alleles (i.e. loci 12 and 21) do not have sufficient diversity amongst the putative parents to specify which parent is mismatched. All surviving offspring from family group 14/09 mismatch the putative father at loci 2 and 21. Kakī conservation practitioners described another male in the area with similar leg bands who paired with the putative mother in subsequent breeding seasons and has alleles that match these offspring; therefore, this mismatch for family group 14/09 is likely the result of human error (i.e. field misobservation). For family group 14/13, one of six offspring (from two clutches) does not match putative parents at loci 2 and 21, with alleles at locus 2 not attributable to the father, and locus 21 having insufficient diversity amongst the putative parents to specify which parent is mismatched.

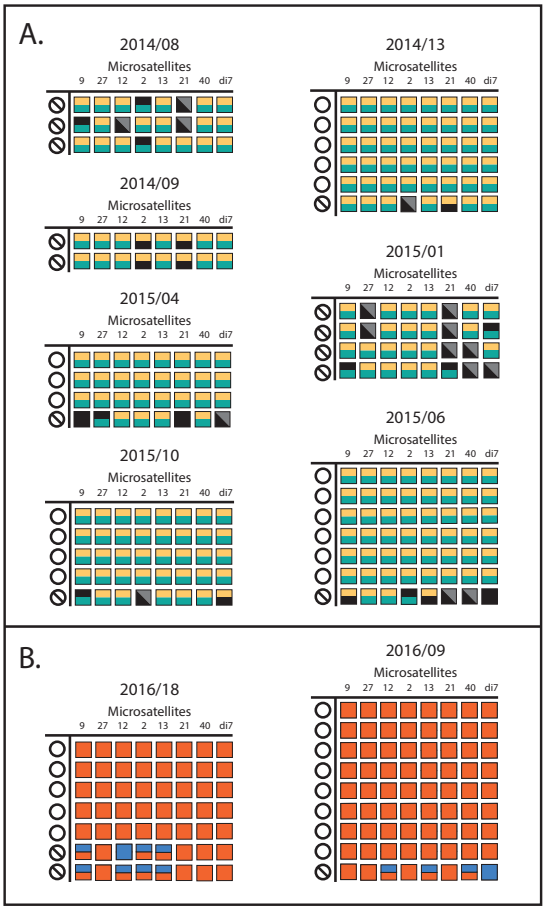

Figure 2. Wild families with offspring excluded by allele-exclusion, including offspring that assign as kakī (A) and non-kakī (B). A) Each offspring is represented by a row with bi-coloured boxes to represent maternal (yellow/top) and paternal (green/bottom) allelic contribution at each locus. Black boxes indicate alleles that could not be attributed to a parent. Boxes with black/gray diagonals indicate mismatch, but insufficient diversity to determine maternal or paternal exclusion. B) Red boxes indicate alleles typical of kakī (all parental alleles), and blue boxes indicate alleles typical of non-kakī (i.e. poaka or kakī x poaka hybrids).

During the 2015 breeding season, there were four family groups that showed alleles that did not correspond between parents and offspring, including family groups with DOC identifiers 15/01, 15/04, 15/06, and 15/10. All four offspring from family group 15/01 have alleles that mismatch the mother (loci 9, 21, and di7) or loci that have insufficient diversity amongst putative parents to specify which parent is mismatched (loci 21, 27, 40, di7). In family group 15/04, one of four offspring mismatches one or both putative parents across loci 9, 21, 27, and di7. For family group 15/06, one individual out of six mismatches from one or both parents across loci 2, 9, 13, 21, 40, and di7. For family group 15/10, one individual mismatches both parents across loci 2, 9, and di7.

During the 2016 breeding season, there were two family groups with alleles which were mismatched from putative parents: family groups 16/09 and 16/18. For family group 16/09, one individual had alleles that are typical for poaka (Steeves *et al*. 2010) and do not assign to either parent. This individual was noted as being atypical prior to analyses, as it was collected only three days after its clutch mates, but hatched a full 10 days later. In family group 16/18, both mismatched individuals were identified as being atypical, as one of their clutches had 5 eggs, as opposed to the typical 4 egg clutch in kakī (Pierce 2013), and their plumage was paler than other juveniles their age. Both pale individuals from family group 16/18 were found to have alleles typical of poaka (Steeves *et al*. 2010) that could not be attributed to either parent.

For all mismatched individuals, the only birds

that did not assign as kakī using STRUCTURE Bayesian clustering analyses were individuals from the 2016 breeding season (assignment probabilities to kakī cluster = 0.21–0.70) from family groups 16/09 and 16/18. Mitochondrial cytochrome *b* for these individuals assign to poaka (node A), as per Steeves *et al*. 2010 (GenBank Accession number: HQ007646).

DISCUSSION

This study is the first to evaluate the kakī pedigree over multiple breeding seasons using genetic markers. Across the 2014–2016 breeding seasons, 5.9% of offspring mismatched with putative parents, including three offspring attributed to non-kakī brood parasitism and two readily explained by human error. These results reinforce current practice to screen atypical kakī nests and suspected introduction of human error to the pedigree, using the methods described here. This study also reveals an opportunity to discuss the factors driving mismatch (see below) and management ramifications of previously unidentifiable error that exists in the kakī pedigree.

Three offspring from the 2016 breeding season displayed microsatellite alleles and mitochondrial sequences typical of poaka that did not correspond to either putative parent. The risk of human error for these misassigned offspring is low, as all eggs collected from the wild for the past 15 years are exclusively gathered from intensively-monitored kakī nests (i.e. all black birds, otherwise known as node J; Steeves *et al*. 2010). Therefore, this genetic data provides strong evidence for ongoing brood parasitism, or egg-dumping, from non-kakī into kakī nests, as described in Overbeek *et al*. (2017). However, unlike Overbeek *et al*. (2017) where suspected egg-dumped individuals were identified by having pale plumage, the egg-dumped individuals from the study here were also identified as they came from nests with atypical life history traits for kakī (i.e. being in clutch of > 4 eggs, or hatching asynchronously with clutch mates). To avoid incorporation of non-kakī into the pedigree and to ensure conservation rearing resources are allocated to kakī only, these combined results indicate that the Kakī Recovery Programme should exclude individuals with atypical plumage or inconsistent life history traits.

Our results also indicate one family group whose mismatched alleles are most easily explained by human error. In family group 2014/09, both offspring have alleles that do not match the recorded father, but do match those of another male recorded in the same area with a similar leg band combination. In addition, the putative mother nested with the latter male in subsequent seasons. Human error is an issue identified in many pedigrees (e.g. dairy cattle *Bos taurus*, Visscher *et al*. 2002; Attwater's prairie-chicken; Hammerly *et al*. 2016; see also Oliehoek & Bijma 2009). This is particularly salient for pedigrees that include wild individuals, where identification can be hampered by leg band misidentification (leg bands are stained, or difficult to observe when birds are wading; e.g. Milligan *et al*. 2003) and when leg bands are dropped due to wear (e.g. Allen *et al*. 2019). To minimise pedigree error that can result from misidentification or transcription issues, we recommend the Kakī Recovery Programme continue to maintain lists of possible human error, periodically screen affected birds accordingly using the approach outlined here, and consider other identification techniques that may reduce error at the nest (e.g. radio frequency identification, or RFID tags; Bonter & Bridge 2011).

Excluding the five offspring readily explained by non-kakī brood parasitism and human error, only 4.1% of offspring studied here have alleles that do not match putative parents and are left unexplained. Although we cannot rule out human error as being the cause for these discrepancies, some offspring have alleles that are suggestive of extra-pair paternity or intraspecific brood parasitism, which has been described in other wild shorebirds (Order: Charadriiformes). This includes Kentish plovers (*Charadrius alexandrinus*) where extra-pair paternity rates are 3.9% (Küpper *et al*. 2004) and common sandpipers (*Actitis hypoleucos*) where extra-pair paternity and intraspecific brood parasitism rates are as high as 15.7% (Mee *et al*. 2004). Research in shorebirds suggests that promiscuous mating behaviour may be more prevalent in social pairs that are closely related as a tactic to avoid negative fitness consequences associated with inbreeding (Blomqvist *et al*. 2002). This scenario resonates with kakī, as the population has experienced inbreeding after a substantial bottleneck (Hagen *et al*. 2011). Other studies suggest that promiscuous mating behaviour and brood parasitism is associated with higher nest densities (Westneat & Sherman 1997). Much of the written behaviour traits described for kakī have been recorded after the population experienced significant decline (i.e. < 200 individuals; Pierce 1984). Therefore, biologists do not know how kakī behaviour may change when they reach higher densities. As the population recovers, comprehensive sampling including all putative parents, combined with an analysis using thousands of single nucleotide polymorphisms, would provide the resolution needed to discern and determine the extent of extra-pair paternity and intraspecific brood parasitism as breeding tactics in kakī.

After examining the explanations for these parentage assignment mismatches, this study has identified a low percentage of error (5.9%) in the kakī pedigree. Given that a simulation study across

domesticated mammals (i.e. cattle; sheep *Ovis aries*; and horse *Equus ferus*) indicates that pedigree error rates >15% could hamper conservation efforts using a mean kinship approach (Oliehoek & Bijma 2009), we consider the utility of the kakī pedigree for conservation genetic management remains high. However, simulation studies tailored to the life history traits of critically endangered species like kakī are likely to provide more informative cut-offs to enable the retention of maximum genome-wide diversity (Galla *et al.* 2020). Should these simulations reveal that even low pedigree error rates inhibit species recovery, the accuracy of the kakī pedigree could be further improved using high resolution single-nucleotide polymorphisms (e.g. Flanagan & Jones 2019). Thus, we recommend the Kakī Recovery Programme further evaluate the implications of pedigree error for the conservation management of this critically endangered taonga species. Beyond kakī, this study highlights the importance of using genetic and genomic technologies to evaluate pedigrees of intensively managed species to better inform conservation management.

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