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RESEARCH

# Multiplex PCR reveals population structure in an inbred communal bird

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**Abstract**: Studying the effects of inbreeding in wild avian populations can be challenging due to attendant concerns and restrictions when working with endangered species. Therefore, naturally inbreeding species listed as Not of Concern, such as the communally breeding pūkeko (*Porphyrio melanotus melanotus*, family Rallidae), may provide an excellent model for experimental analyses of inbreeding. We hope that this work will eventually inform conservation efforts directed at endangered species. We sampled pūkeko populations on the North Island (Tāwharanui Park) and South Island (Otokia Reserve) of Aotearoa New Zealand that differ in climate and breeding behaviour. North Island populations are philopatric, living on year-round territories, and in kin groups, leading to inbreeding once the young become breeders. South Island populations have seasonal territories, high dispersal rates, and form non-kin groups, resulting in outbreeding. Given behavioural evidence of inbreeding at a nearby North Island population, we predicted that the North Island population would exhibit lower heterozygosity and higher inbreeding coefficients than the South Island population. To test these predictions, we developed microsatellite primers, optimised multiplex PCRs, and genotyped breeding groups from the North and South Island. In this pilot study we found that breeding groups from North Island were genetically differentiated, whereas population structure was not detected in the South Island groups. North Island birds also had higher inbreeding coefficients and levels of within-group kinship than South Island birds. This pilot study validated microsatellite markers and multiplex PCR methods and is, to our knowledge, the first genetic analysis of population structure and relatedness within communal breeding pūkeko. These genetic tools will be used for larger-scale studies to understand interactions between breeding behaviour and inbreeding.

## Introduction

Most endangered species have small and declining populations (Wilcove et al. 1993), which increases inbreeding. Inbreeding occurs when close kin reproduce (Blomqvist et al. 2010). In some cases, this results in inbreeding depression and may contribute to the extinction vortex (Blomqvist et al. 2010), which is when the reduction in population size increases inbreeding depression, resulting in further population decreases and continued inbreeding (Brook et al. 2002). Endangered species in small populations are typically protected and research efforts that may harm them are prohibited. However, species in large and growing populations that naturally engage in inbreeding can be used to investigate inbreeding depression (Kardos et al. 2016), which then may inform endangered species conservation efforts.

Inbreeding decreases heterozygosity and may lead to inbreeding depression (Keller & Waller 2002). Inbreeding depression is thought to stem from two sources. The first is that fitness declines when deleterious recessive alleles are more frequently homozygous because of inbreeding (Keller & Waller 2002). The second is that fitness may decline when advantageous heterozygous genotypes are less prevalent. Species may avoid inbreeding through natal dispersal, sometimes by only one sex. This reduces risks of inbreeding if breeding occurs after dispersal (Pusey & Wolf 1996). Nonetheless, inbreeding is still observed in healthy wild populations despite potential negative effects (Keller & Arcese 1998; Langen et al. 2011). One potential benefit of inbreeding is that it may facilitate the purging of deleterious recessive alleles that would otherwise be less susceptible to removal in an outbred population because they would often be heterozygous with a wild type allele (Hedrick 1994; Keller & Waller 2002). However, selection against deleterious recessive alleles also decreases population size by decreasing the fitness of members that inherit two deleterious recessive alleles, which itself is a factor that can increase inbreeding (Langen et al. 2011).

Interestingly, a recent study found that animals rarely avoid mating with kin and identified publication bias favoring papers showing inbreeding avoidance (de Boer et al. 2021). Under conditions in which inbreeding is associated with purging of deleterious recessive genes, there may be indirect fitness benefits from inbreeding because of increased kin-shared alleles (Langen et al. 2011). More generally, the effects of inbreeding may vary over time and among species and ecological contexts,

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and why, when, and with whom inbreeding occurs is not always apparent (Keller & Waller 2002). Thus, it is important to study inbreeding and its consequences in a diversity of organisms and contexts. This is especially important if studies of non-endangered species have the potential to inform our understanding of inbreeding depression in small populations of endangered species.

Pūkeko (*Porphyrio melanotus melanotus*, family Ralliade) are communally breeding birds found in eastern Australia and New Zealand. In New Zealand, pūkeko exhibit cooperative, polygynandrous breeding and joint laying, where members of a breeding group share a communal nest that multiple females lay eggs into, and breeding individuals mate with multiple breeding partners (Craig 1980; Jamieson 1997). We studied pūkeko from New Zealand's North Island (Tāwharanui Regional Park) and South Island (Otokia Wildlife Reserve; Fig. 1). The North Island population experiences a milder climate than the South Island population, allowing them to defend year-round territories (Jamieson 1997). Pūkeko populations on the North Island have low natal dispersal (Shakespear Regional Park, Jamieson 1997; Tāwharanui Regional Park, Dey et al. 2014), likely due to habitat saturation that limits dispersal options. Stable groups include one to seven breeding males, one to two (or three, JSQ, pers. obs.) breeding females, and non-breeding helpers (Jamieson 1997). Observational data (collected at Shakespear Regional Park in the North Island; Fig. 1) indicate a high frequency of inbreeding, often between first-degree relatives (Craig & Jamieson 1988), likely due to a combination of year-round territoriality and philopatry, and high reproductive skew in North Island populations (Jamieson 1997). By contrast, birds in the South Island population experience a harsher climate and changes in food availability in the winter, forcing them to abandon territories annually (Jamieson 1997). During the non-breeding season, behavioural data indicate that South Island birds join large foraging flocks and re-establish breeding groups in new configurations of group members each breeding season, without helpers, resulting in non-kin breeding groups that lack inbreeding (Jamieson 1997). South Island group sizes are smaller with one to three males, one or two females, and no helpers (Jamieson 1997).

Overall, a high degree of kinship and inbreeding has been documented by demographic and behavioural observations

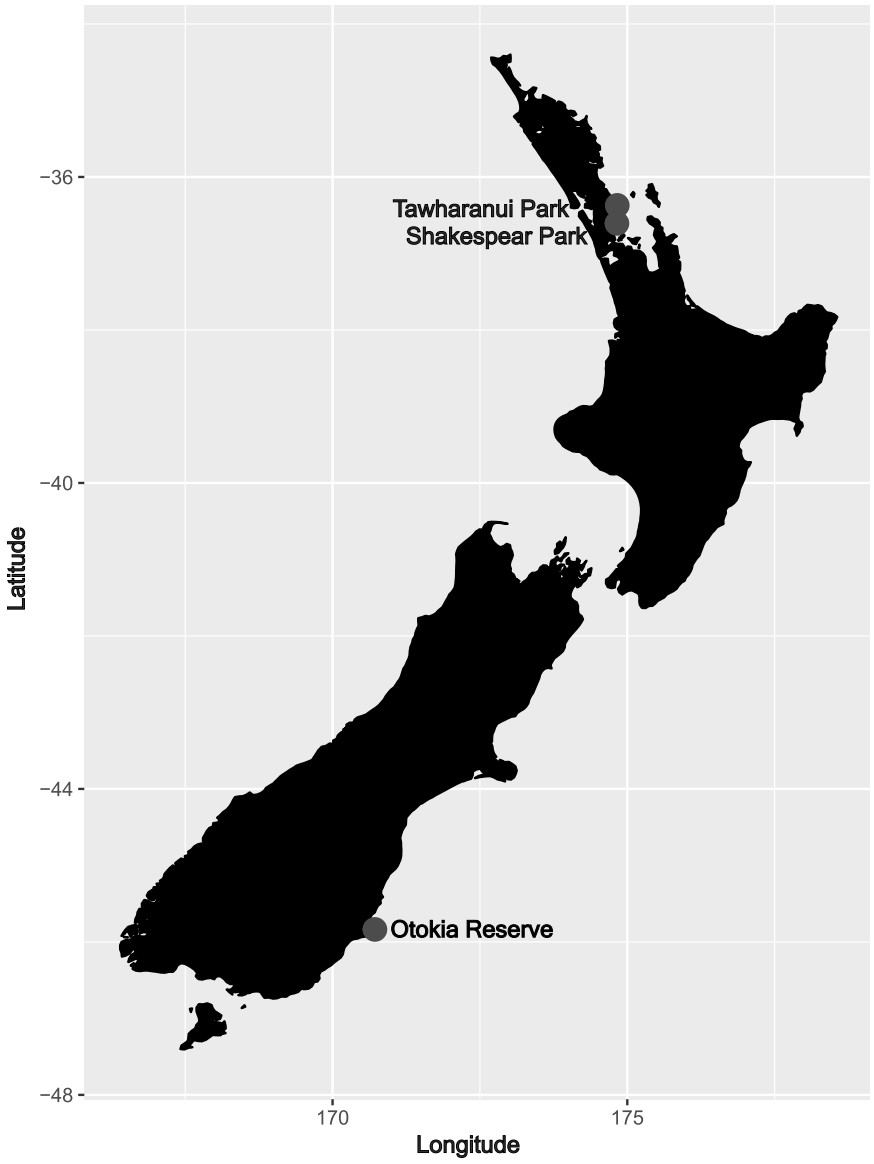


**Figure 1**. Map of New Zealand showing locations of North Island populations (Tāwharanui and Shakespear) and a South Island population (Otokia). Figure generated using ggplot2 and mapdata R packages (Wickham 2016; Becker and Wilks 2018).

in North Island populations (Craig & Jamieson 1988). The fitness consequences of this, however, are still unknown and there are limited genetic studies of the species. It is important to understand the degree to which inbreeding in the North Island populations has reduced within group heterozygosity and to what extent this causes inbreeding depression. This can be achieved by comparing North Island and South Island populations. Pūkeko are listed as Not Threatened by the New Zealand Department of Conservation and Least Concern' by the International Union for Conservation of Nature, making them tractable to study. The contrast between the North Island population, with nonbreeding helpers on year-round territories, and the South Island population, with outbred breeding groups on seasonal breeding territories without helpers, makes pūkeko a compelling model for exploring effects of inbreeding and kinship on inclusive fitness and population stability. To that end, we developed molecular tools for pūkeko. Our primary goal was to develop microsatellite markers and optimise multiplex PCR protocols that will facilitate large scale population genetic analyses in this study system. Additionally, because of the polygynandrous mating system and the likelihood that some matings involve non-kin, we anticipate future research opportunities to monitor the fate of inbred and outbred eggs in the same joint nest, enabling us to measure the effects of inbreeding on growth, survival, and fitness.

Previous genetic analyses of pūkeko used resource intensive techniques (Southern blotting with minisatellite probes) that were of lower genetic resolution and were difficult to assess statistically (Jamieson et al. 1994; Lambert et al. 1994). Here we describe the development of 18 polymorphic microsatellite loci and a pilot study in which we compared inbreeding coefficients between the North Island and South Island populations, testing for genetic differentiation among breeding groups within populations. Based on prior behavioural evidence of inbreeding in a North Island population at Shakespear Regional Park located about 27 km south of Tāwharanui Regional Park (Craig & Jamieson 1988; Fig. 1) and seasonal mixing of breeding groups in the South Island population (Jamieson 1997), we predicted higher inbreeding coefficients and greater within breeding group kinship in North Island (Tāwharanui) than South Island (Otokia). We predicted significant genetic structure based on breeding group differentiation within the North Island, but not South Island, based on a lack of local migration or membership exchange among breeding groups in the north.

## Methods

### Sample collection

We used blood samples collected as part of long-term research on pūkeko. We selected four breeding groups from the Tāwharanui park North Island population (HAY1N: $n$ = 7, NPBS: $n$ = 9, NPBE: $n$ = 5, RFSE: $n$ = 6) sampled in 2010 by Cody Dey and James S. Quinn and two breeding groups from the Otokia South Island population (W1: $n$ = 4, W2: $n$ = 4) sampled in 1991 and 1992 by Ian Jamieson (Jamieson et al. 1994). For this pilot study we opportunistically selected breeding groups for which we had high quality DNA (see Appendix S1 in Supplementary Material).

### Microsatellite identification

Using one third of a lane of an Illumina Hiseq X 2500 machine, we collected short read data (150 base pair (bp) paired-end sequences) from three female pūkeko with barcoded DNA (one each from Tāwharanui park [North Island], Shakespear park [North Island], and Otokia [South Island]), prepared by The Centre for Applied Genomics (Toronto, Canada). We used PEAR (Zhang et al. 2014) to merge paired end sequences, and MISA (Thiel et al. 2003; Beier et al. 2017) with a Perl script to identify tetrameric microsatellites with at least 40 bp flanking sequence on each side of the repeat for primers. These raw data have been deposited in the NCBI Sequence Read Archive (BioProject doi: accession PRJNA1073884).

### Primer design, optimisation and genotyping

Primers were designed in Primer3 using tetramers with at least eight repeats (Koressaar & Remm 2007; Untergasser et al. 2012; Koressaar et al. 2018) based on the Illumina paired-end sequences described above. For multiplex PCR, which involves amplifying multiple loci in the same reaction, and subsequent genotyping, each forward primer was labeled at the 5' end with fluorescent labels (HEX and 6FAM, Integrated DNA Technologies; NED, Thermo Fisher Scientific). PCR was performed in 25 µL reaction volumes containing nuclease-free water, 1X PCR reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen by Thermo Fisher Scientific), 0.2 µM of each forward and reverse primer pair, 0.5 U Taq polymerase (Thermo Fisher Scientific), and 1 µL template DNA (extracted from pūkeko blood samples using a standard salt extraction procedure; Miller et al. 1988). We used a thermocycling program of 3 min initial denaturation at 95°C, followed by 30 cycles of 30 sec denaturation at 95°C, 30 sec annealing at (50–60°C; see Appendix S2), 45 sec extensions at 72°C, and a 10 min final extension at 72°C. All reactions were performed using an MJ Research PTC-200 Thermocycler. Where possible, individual primer pairs were multiplexed in groups of three.

After PCR, we mixed 5 µL of PCR product with 1 µL of 6X loading dye (Thermo Fisher Scientific) and ran samples on 2% agarose gels stained with RedSafe (FroggaBio) at 95 V for 30 mins and visualised under UV light (Bio-Rad ChemiDoc Imaging System, Image Lab) to confirm amplification at the expected allele size. PCR products were then loaded into 96-well plates, cleaned using an in-house ethanol precipitation PCR cleanup protocol (Appendix S3), and sent for genotyping on a 48-capillary ABI 3730 at the Trent University Wildlife Forensic DNA Laboratory (Peterborough, Canada). Overall, we genotyped 36 individuals from the North Island and 13 individuals from the South Island at 22 potential microsatellite loci (Appendix S1). Out of the 22 primers we optimised, four were unusable. For primers TAWH49 and TAWH33, electropherogram peaks could not be scored as microsatellites and were discarded. Primers TAWH7 and TAWH139 were monomorphic, only showing peaks at 174 bp and 210 bp respectively, and this was observed in every individual that had amplification at these loci ($n$ = 8 and 30 respectively), so these primers were also discarded. Primer TAWH46 had consistent non-target amplification at 124 bp in almost all individuals so this peak was excluded, but we observed clear peaks at 150 or 154 bp that were retained and scored as microsatellites. Ultimately, we retained 18 microsatellite loci for analysis. Table 1 includes the primer sequences, fluorescent labels, annealing temperatures for each microsatellite locus included in this study, expected product sizes, number of alleles detected, and multiplex groupings.

**Table 1.** Details for each working multiplex, including primer name (locus), sequence, fluorophore added to 5' end of primer, expected product size, primer melting temperature (Tm), number of alleles detected in each population, and whether the locus was retained for further analysis. N/A means that a population was not genotyped at that locus.

| | Locus | Primer Sequence | Fluorophore | Expected Size (bp) | Tm (°C) | No. of Alleles (North, South) | Retained? |
|---|---|---|---|---|---|---|---|
| Multiplex 1 | TAWH226 | F: aggacaggcaggattaagatga<br>R: aagaggagggagggaaggaa | NED | 116 | 55.5 | 2, 5 | Yes |
| | TAWH252 | F: gcactggagcgttagtacca<br>R: tggattctcgggtatgtagct | HEX | 156 | 55.5 | 4, 3 | Yes |
| | TAWH111 | F: cgctagggaaatgggctcta<br>R: ttgcttgacagtggaattacttc | FAM | 243 | 55.5 | 3, 6 | Yes |
| Multiplex 2 | TAWH49 | F: aactgaacagacacatgcct<br>R: accatcactagttcctcctgc | NED | 150 | 55 | non-microsatellite | No |
| | TAWH7 | F: tgcagacgaggtgtaatagaga<br>R: tagctgagtctggaggtgga | HEX | 216 | 55 | 1 (monomorphic) | No |
| | TAWH240 | F: cccttccaacctagaccagt<br>R: gtcacttcccctctacccag | FAM | 245 | 55 | 5, 4 | Yes |
| Multiplex 3 | TAWH269 | F: cataagaaagccagaaccaaagt<br>R: gtttgctgtattccttagggca | HEX | 247 | 59.3 | 4, 5 | Yes |
| | TAWH53 | F: ctctcacagcagcaggtttg<br>R: ggatgtcctgacctgtcctc | NED | 200 | 59.3 | 5, 4 | Yes |
| | TAWH104 | F: cttgggtggttaaggggct<br>R: cgacagacagacagaggct | FAM | 150 | 59.3 | 3, 4 | Yes |
| Multiplex 4 | TAWH33 | F: aaccaaatctcatgctttccag<br>R: aggcagtcaatttgtagcagt | FAM | 247 | 60 | non-microsatellite | No |
| | TAWH191 | F: cccactctgtttaactttctggg<br>R: agctaaagaatgatacagcagca | HEX | 101 | 60 | 3, 2 | Yes |
| | TAWH46 | F: caggagggtttgcagacttg<br>R: tcacatcctcagagagcagc | NED | 150 | 60 | 2, 4 | Yes |
| Multiplex 5 | TAWH84 | F: tggcacaggaaggtatcagg<br>R: gatggtccctgtggtggtg | FAM | 246 | 59.5 | 7, 6 | Yes |
| | TAWH223 | F: atggacagacggacagacag<br>R: ctagtgtgggcctactcctg | NED | 112 | 59.5 | 3, 2 | Yes |
| | TAWH138 | F: caagagcccagagttacagc<br>R: agtgatgtaagtgggactcagg | HEX | 207 | 59.5 | 2, 1 | Yes |
| Monoplex | TAWH182 | F: caaggctctgctcatgtgtt<br>R: cctcctttgacattgctggc | FAM | 242 | 56 | 5, 4 | Yes |
| Monoplex | TAWH280 | F: tctttgtagctgggttttggg<br>R: ttttgcaggttgacagctca | HEX | 160 | 58 | 3, 4 | Yes |
| Monoplex | TAWH3 | F: cacgtggctctggatctcc<br>R: agttactcaatgggctgcct | FAM | 233 | 60 | 3, N/A | Yes |
| Monoplex | TAWH171 | F: agcagcattcagagcccata<br>R: ttggccagaaagagaaacgg | FAM | 206 | 56.3 | 2, 2 | Yes |
| Monoplex | TAWH199 | F: cctgccaagtctatctaccca<br>R: aatgggctgtttggtggttg | HEX | 166 | 56 | 3, 3 | Yes |
| Monoplex | TAWH14 | F: gacagacagacggagggatg<br>R: atctgtccatcgtcttcccc | HEX | 249 | 52 | 3, 2 | Yes |
| Monoplex | TAWH139 | F: caagagcccagagttacagc<br>R: agtgatgtaagtgggactcagg | FAM | 207 | 52 | 1 (monomorphic) | No |

### Statistical Analysis

To genotype the microsatellites, we scored electropherograms using PeakScanner 2.0 using recommended methods in the Applied Biosystems DNA Fragment Analysis by Capillary Electrophoresis manual (2012). We used Arlequin v.3.2.2 (Excoffier & Lischer 2010) software for population genetic analyses, including calculations of Hardy-Weinberg equilibrium (HWE), pairwise $F_{ST}$, and analyses of molecular variance (AMOVA). We used AMOVA to test for evidence of genetic structure within populations and to calculate conventional F-statistics; pairwise $F_{ST}$ was used to evaluate population structure between breeding groups within populations. A Mantel test was used to test for isolation-by-distance and was run with 999 permutations using the R package vegan (Oksanen et al. 2022). To correct for multiple tests, we applied a Bonferroni correction for AMOVA tests and pairwise $F_{ST}$ using the stats base package in R as described by Rice (1989). The significance of global $F_{ST}$ and $F_{IS}$ between populations was tested by comparing bootstrap confidence interval values in a paired, two-tailed t-test. To visualise genetic differences between breeding groups and populations, we ran PCAs on raw allele counts using the R package adegenet (Jombart 2008), generating biplots of allelic similarity. Relatedness between individuals was investigated using COANCESTRY v.1.0.1.10 using a moment estimator (Wang 2002; Wang 2011). Heatmaps were generated using the ggplot2 package in R (Wickham 2016).

## Results

### Multiplexes

We successfully optimised five multiplexes, each with three primer sets, and six additional monoplexes (Table 1) for a total of 22 microsatellite loci. Each primer pair also amplified reliably in monoplex using the same PCR conditions as reported for multiplex reactions (Appendix S2). Genotyping multiple individuals at each locus revealed allelic polymorphism in 18 microsatellites, 2 monomorphic microsatellites, and 2 unscorable microsatellites (Table 1). TAWH49 and TAWH33 may produce scorable results in monoplex, even though we did not obtain useable data in multiplex.

### Hardy-Weinberg equilibrium tests

For all 18 polymorphic microsatellite loci and six breeding groups genotyped there were only three loci (TAWH53, TAWH269, TAWH223) that departed significantly from Hardy-Weinberg equilibrium **(**HWE) expectations. Each departure was for only one breeding group (Appendix S4) and each was due to a deficiency of observed heterozygotes. Within the North Island population, the HAY1N breeding group had four monomorphic loci and two loci that were out of HWE, with observed heterozygosity significantly lower than expected (Appendix S4). The NPBS breeding group had six monomorphic loci and no loci deviating significantly from HWE. Lastly, RFSE and NPBE groups each had seven monomorphic loci and none departed significantly from HWE. The South Island population had two monomorphic loci and one locus that deviated from HWE due to lower-than-expected heterozygosity. Breeding groups W1 and W2 each had three monomorphic loci, two of which were the same loci. Group W1 had one locus that deviated significantly from HWE, while W2 had none (Appendix S4).

### Population structure and relatedness

We found significant differentiation among breeding groups within the North Island population, with an $F_{ST}$ value of 0.24 (probability of non-departure from zero < 0.01; Table 2a). Pairwise $F_{ST}$ scores among North Island breeding groups were all significant using Bonferroni corrected p values (Table 3). $F_{ST}$ values contrasting dyads of breeding groups from North Island were not significantly correlated with the distances between the nests of those groups in the dyads (Mantel test, $p = 0.71$).

No population subdivision was detected between the two South Island breeding groups ($F_{ST} = 0$; $p = 1.00$; Table 2b). Pairwise $F_{ST}$ scores between South Island breeding groups was 0.005, which was not significantly different from zero ($p = 0.92$; Table 3). To compare F-statistics for the two populations, we used 95% confidence intervals. The $F_{ST}$ and $F_{IS}$ values at North Island were both significantly higher than South Island ($p < 0.0001$, Table 2a&b). Unexpectedly, $F_{IS}$ values for the South Island samples were statistically significant (Table 2b).

A PCA contrasting North and South Island populations indicated they cluster separately, with little to no overlap (Fig. 2). Within North Island, HAY1N clusters the farthest from other breeding groups, with NPBE and NPBS clustering closer to RFSE. The two South Island breeding groups overlap with each other.

As expected, relatedness within breeding groups was higher in the North Island population than the South Island population (Fig. 3). Individuals in North Island breeding groups had higher coefficients of relatedness ($r = 0.5–1.0$), whereas between breeding groups, relatedness was comparatively lower (Fig. 3). South Island breeding groups did not have a high coefficient of relatedness, apart from one dyad with $r = 0.5$ (see Appendix S5 for dyad relatedness values and 95% confidence intervals).

## Discussion

We successfully identified 18 polymorphic microsatellite markers for pūkeko, designed PCR primers that amplified their alleles, and used these data in a pilot test for population genetic verification of patterns that were discovered based on demographic and behavioural data from breeding groups that occupied year-round territories at Tāwharanui and Shakespear regional parks in the North Island. The genetic results reported here represent a pilot study that will be followed by a study of over 1000 adult blood samples collected since 2008 from > 60 North Island (Tāwharanui) groups. The main goal of this research was to establish a set of genetic tools for the study of parentage and kinship, as well as the genetic consequences of philopatry and year-round territoriality. These 18 newly developed polymorphic microsatellites will allow us to study the consequences of inbreeding on individuals, breeding groups, and populations that are currently healthy and readily available for study. Three loci were significantly out of HWE in one of the six breeding groups (Appendix S4). These three loci may have null alleles, which we were unable to explore in this study due to low sample sizes but will explore in the future.

We used these new microsatellite loci to test for genetic structure in four breeding groups from the Tāwharanui population on the North Island of New Zealand and two groups from the Otokia population on the South Island. While other studies have shown genetic structuring of cooperative breeding groups (see below), ours is the first study of a species

**Table 2.** North Island (Tāwharanui) and South Island (Otokia) whole population AMOVA tests. Global AMOVA results are expressed as a weighted average over polymorphic loci. P-values adjusted using Bonferroni correction. Significance was tested using randomization tests with 15 000 permutations and 95% confidence intervals (CI) are indicated.

| **North Island (*n* = 27 adults).** | | | |
|---|---|---|---|
| **Source of variation** | **Sum of squares** | **Variance components** | **Percentage genetic variation** |
| Among breeding groups (*n* = 4) | 79.8 | 1.44 | 23.8 |
| Among individuals within breeding groups | 161.3 | 2.11 | 34.9 |
| Within individuals | 70.0 | 2.50 | 41.3 |
| FIS = 0.458 CI: 0.329–0.595<br>FST = 0.238 CI: 0.141–0.342<br>FIT = 0.587 CI: 0.479–0.700 | | | |
| FIS: p = 0.000<br>FST: p = 0.000<br>FIT: p = 0.000 | | | |
| **South Island (*n* = 8 adults)** | | | |
| **Source of variation** | **Sum of squares** | **Variance components** | **Percentage genetic variation** |
| Among breeding groups (*n* = 2) | 5.25 | −0.24 | −4.66 |
| Among individuals within breeding groups | 42.90 | 1.82 | 35.84 |
| Within individuals | 28.00 | 3.50 | 68.80 |
| FIS = 0.342 CI: 0.148–0.538<br>FST =−0.047 CI:−0.107–0.014<br>FIT = 0.312 CI: 0.131–0.502 | | | |
| FIS: p = 0.000<br>FST: p = 1.000<br>FIT: p = 0.000 | | | |

**Table 3.** Pairwise $F_{ST}$ for comparisons between and within North Island (Tāwharanui; HAY1N, NPBS, NPBE, RFSE) & South Island (Otokia; W1, W2) breeding groups. P-values adjusted using Bonferroni correction. Green: significant at alpha = 0.05; red: non-significant. Departure from the null hypothesis of FST being equal to zero was assessed using 15 000 permutations. Only comparisons between breeding groups in the same location are reported.

| | **HAY1N** | **NPBS** | **NPBE** | **RFSE** | **W1** | **W2** |
|---|---|---|---|---|---|---|
| **HAY1N** | 0.00 | | | | | |
| **NPBS** | FST = 0.191<br>p = 0.006 | 0.00 | | | | |
| **NPBE** | FST = 0.306<br>p = 0.002 | FST = 0.326<br>p = 0.006 | 0.00 | | | |
| **RFSE** | FST = 0.297<br>p = 0.018 | FST = 0.294<br>p = 0.006 | FST = 0.144<br>p = 0.024 | 0.00 | | |
| **W1** | | | | | 0.00 | |
| **W2** | | | | | FST = 0.005<br>p = 0.915 | 0.00 |

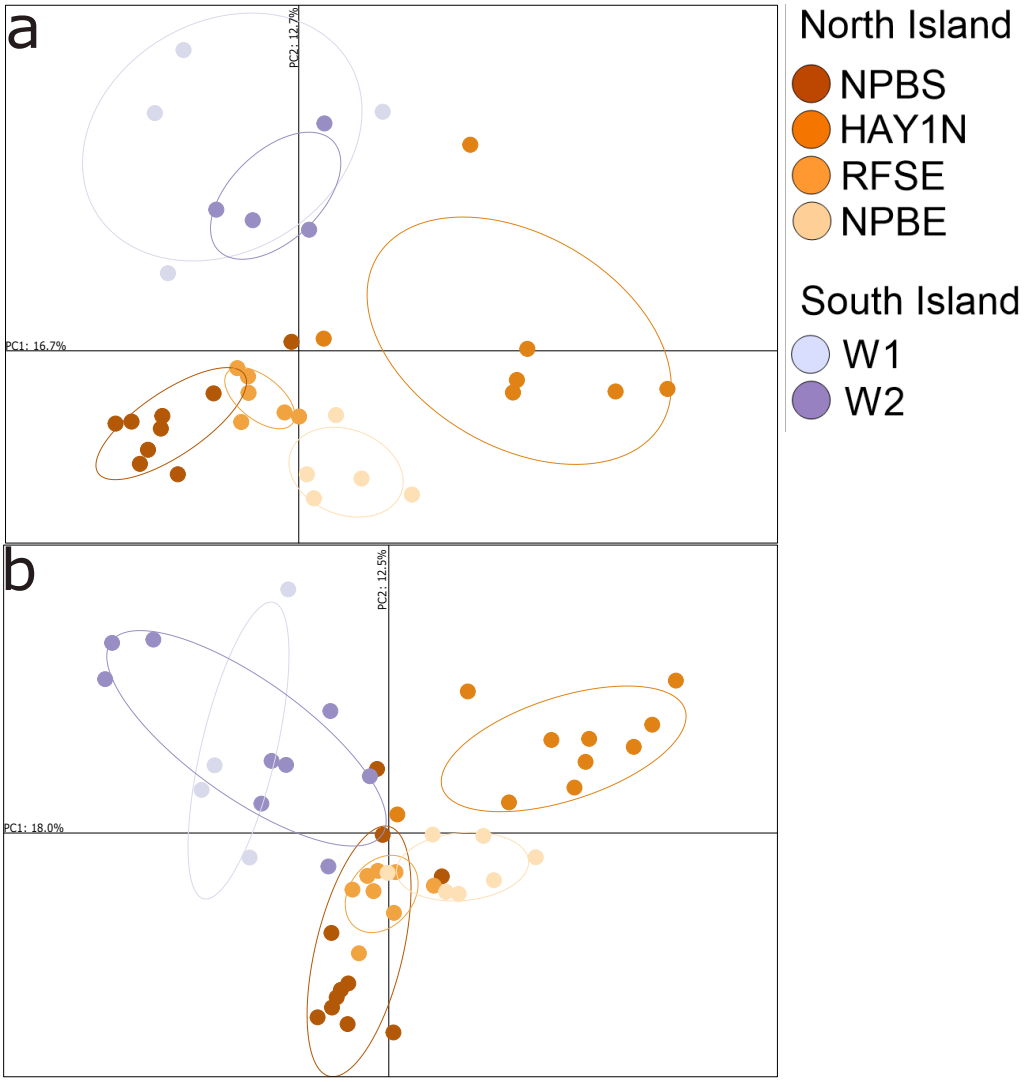


**Figure 2**. PCA of breeding groups from North Island (oranges) and South Island (purples) populations (a) excluding offspring (b) including offspring. Ellipses of inertia show where the majority of individuals cluster for each group. Figure generated using the adegenet package in R (Jombart 2008) and legends were generated in GraphPad Prism version 10.0.0. for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

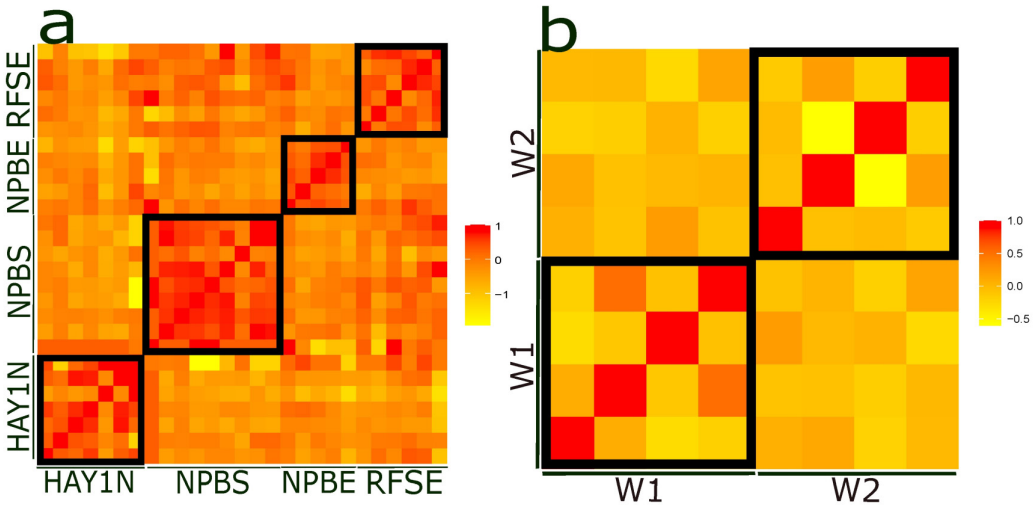


**Figure 3**. Heat maps depicting dyadic coefficients of relatedness (a) North Island (Tāwharanui) breeding groups (b) South Island (Otokia) breeding groups. Bold outlines indicate within breeding group comparisons.

with co-breeding males and females. This system, structured by breeding groups in the North Island population, promotes frequent inbreeding unless strong inbreeding avoidance mechanisms are in place.

**Genetic structure, group kinship, and inbreeding**

Breeding groups in the North Island are genetically differentiated from each other based on AMOVA analysis (Table 2) and significant positive $F_{ST}$ values for all pairwise comparisons (Table 3). The mostly non-overlapping grouping of individuals in each breeding group in the North Island, but not South Island populations in a principal component analysis (Fig. 2) is consistent with the $F_{ST}$ results. This was expected given field observations that neither offspring nor adults relocate between territories within Tāwharanui park in the North Island (pers. obs. James S. Quinn). Over the years of study since 2008, we found one banded individual, discovered by chance, that had migrated from our North Island study site. That bird dispersed over water (Kauwa Bay) approximately 10.5 km to Scandrett Regional Park. We have not yet systematically studied migration away from the North Island study site.

Inbreeding coefficients ($F_{IS}$ values) partially supported our prediction that birds from North Island are more inbred than those from South Island. The North Island population $F_{IS}$ values were significantly higher than those for the South Island population, supporting our prediction (Table 2). However, unexpectedly, the $F_{IS}$ values in South Island were statistically significant, (Table 2b).

Microsatellite genotyping is prone to null alleles, which occur when an allele fails to amplify during PCR, sometimes due to mutations not allowing for primers to bind (Dakin & Avise 2004). This leads to an underestimation of heterozygosity and a consequent overestimation of homozygosity. It is possible that null alleles caused an overestimate of inbreeding ($F_{IS}$) at both sites (Appendix S5). For example, in the North Island, we observed 4–7 monomorphic loci in samples from each breeding group. In the South Island, we observed three monomorphic loci in each breeding group, but did not expect inbreeding. Null alleles may have caused an overestimate of homozygosity in our samples, leading to a less precise estimation of inbreeding (Chapuis & Estoup 2007; Waples 2018), however we do not expect that the significant difference between sites is an error. Due to expected inbreeding at the North Island, we were unable to detect the presence of null alleles using deviations from Hardy-Weinberg Equilibrium. Thus, null alleles would have to be detected in the South Island, which we are also unable to do due to small samples sizes at this site. Future studies should test for null alleles prior to drawing conclusions from data.

We predicted that pūkeko from the North Island would show signals of higher kinship resulting from strong philopatry and more frequent kin-matings within breeding groups. Indeed, we found that the North Island population was structured by breeding group differentiation, based on significant $F_{ST}$ values. Additionally, high coefficients of relatedness were common within, and much less so between, breeding groups in the North Island and not South Island (Fig. 3). This pattern matches demographic and behavioural observations (Jamieson 1997; JSQ, pers. obs.). It appears that high levels of inbreeding in groups on year-round territories has led to a structured North Island population with frequent close kinships among group members (Wang & Shete 2017). We do not have evidence for genetic structuring in the South Island at Otokia, nor did we expect to find it there, but we acknowledge that our pilot analysis has small sample sizes and is based on only two breeding groups.

High relatedness coefficients within each of the four North Island breeding groups (Fig. 3) suggest heightened potential for inbreeding. Having multiple male and female breeders in groups of polygyandrous pūkeko makes this example distinct from other cooperative breeding systems in terms of genetic structure. Several other cooperative breeding bird species show similar patterns of genetic structure based on social groups (e.g., apostle bird, *Struthidea cinerea*, Woxvold et al. 2006; bell miner, *Manorina melanophrys*, Painter et al. 2000; white-browed sparrow-weaver, *Plocepasser mahali*, Harrison et al. 2014; white-winged chough, *Corcorax melanorhamphos*, Leon et al. 2022). Another cooperative breeding species (superb fairy-wren, *Malurus cyaneus*) does not show similar genetic structure between groups (Double et al. 2005), but this species has a very complex social system that includes common extrapair paternity outside the social group (Mulder et al. 1994).

Our analysis, a pilot study of the population genetics of this unique species, comes with limitations. Most notably, we observed higher-than-expected $F_{IS}$ values in the South Island population, despite a lack of evidence for inbreeding. Because of this and the high numbers of monomorphic loci within breeding groups (especially in the South Island; Appendix S4), we suspect that null alleles may be responsible for the high $F_{IS}$ values in that population. We will explore the possible null alleles using ML-NullFreqfrequency and other software (Dąbrowski et al. 2015) with a larger data set to determine whether they are real, and if so, how many actual null alleles there were. Another limitation to our analysis is the small sample sizes, which limits our ability to draw definitive conclusions about each population. For population genetic studies using microsatellites, 25–30 individuals from each population should be included for accurate estimates (Hale et al. 2012).

## Conclusions

We conducted this pilot study to identify microsatellites, design new primers to amplify them, and test them on samples of wild caught pūkeko. We successfully designed five multiplexes, each containing three primer pairs, and 22 microsatellites were individually assayed. In total, we had 18 polymorphic loci, two monomorphic, and two unscorable microsatellite loci for pūkeko. One multiplex contained two loci that were unscorable in multiplex, but that may be scorable in monoplex. Using the 18 polymorphic loci, we estimated heterozygosity and inbreeding coefficients within and between breeding groups and between the North and South Island study populations. These robust protocols will be used by future researchers engaged in pūkeko population genetics studies and will also facilitate future investigations of kinship and parentage analyses in pūkeko.

Results from this pilot study begin to explore differences between North and South Island New Zealand pūkeko populations using microsatellites by determining patterns of heterozygosity and inbreeding coefficients in a North Island pūkeko population with high levels of kin-mating compared with an outbred South Island population. Our analyses suggest that the North Island population lives in a structured population of inbred kin groups living and breeding on year-round territories, while the South Island population lives in a non-structured outbred population living on seasonal territories and mixing in the non-breeding season, consistent with behavioural analyses (Craig & Jameison 1990; Jamieson 1997). Our pilot study presents preliminary genetic analyses suggesting that it

may be useful to study pūkeko as a model for wild inbreeding populations that will help us understand the fitness impacts of inbreeding in declining endangered populations and how inbreeding depression and loss of heterozygosity contributes to fitness decline.

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## Additional Information and Declarations

**Author contributions***:* All authors read and approved the manuscript. Conceptualization: JSQ. Methodology: SB, Formal analysis and investigation: SB, BE, LAG, JSQ; Writing – original draft preparation: SB; review and editing: JSQ, LAG, BE; Funding acquisition: JSQ; Supervision: JSQ.

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**Data availability***:* The accession number for the short read Illumina sequence data upon which our primers are based has been deposited in the NCBI SRA (PRJNA1073884) and the perl and R-code and data input files for analysis can be accessed through GitHub (https://github.com/SarahBabaei/Population-Structure-in-an-inbred-communal-bird).

**Ethics***:* The study and its protocols and methods were approved by Ngāti Manuhiri. Field research methodology, including capturing and blood sampling of pūkeko adults and chicks, was approved by Massey University Animal Care Committee AEC/12 19-9-2008 and McMaster University Animal Research Ethics Board (#13-10-37). Field research was approved by the New Zealand Wildlife Act Authority for Wildlife not located on public conservation land; authorisation number 39641-FAU, and work in Tāwharanui park was approved by Auckland Council (permit NS257). Banding and taking blood samples from pūkeko was approved by the New Zealand Bird Banding Office (Permit No. 2008/066). Access to the Field site in Otokia (South Island) from 1990 to 1992 was granted to Ian Jamieson (Otago University) by Otago Fish & Game and New Zealand Department of Conservation. Exporting of blood samples in lysis buffer to Canada was approved by the New Zealand Ministry for Primary Industries.

**Conflicts of interest**: The authors declare no conflicts of interest.

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## Supplementary Materials

Additional supporting information may be found in the online version of this article:

**Appendix S1:** Table of each individual from each breeding group.

**Appendix S2:** PCR recipe and thermocycling conditions for each multiplex/monoplex.

**Appendix S3:** PCR cleanup protocol using ethanol precipitation.

**Appendix S4:** Hardy-Weinberg test values for each breeding group at each locus.

**Appendix S5:** Coancestry relatedness values and 95% confidence intervals.

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