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SHORT NOTE

Combining morphometric and molecular approaches improves accuracy of sexing in the kakerori (*Pomarea dimidiata*) on Rarotonga, Cook Islands

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The kakerori or Rarotonga monarch (*Pomarea dimidiata*; Monarchinae) is an endangered passerine endemic to Rarotonga in the southern Cook Is, South Pacific. The species was widespread and common on Rarotonga in the mid-1800s, but since the arrival of ship rats (*Rattus rattus*) the population declined to a low point of 29 birds by 1989 (Robertson *et al.* 1994). Intensive management efforts, based on rat poisoning within 150 ha of the Takitumu Conservation Area, has helped the kakerori population in Rarotonga to recover to around 250 birds at present (Robertson *et al*. 1994, Saul *et al*. 1998, Robertson & Saul 2008). To further lower the extinction risk of this single-island endemic on Rarotonga, which is thought to be highly vulnerable to catastrophic events such as cyclones, new diseases, and predators, an insurance population was established on the island of Atiu by translocation of 30 young birds (10 birds per year) from Rarotonga between 2001 and 2003 (Robertson *et al*. 2006).

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The population of kakerori on Rarotonga has been followed since 1987 through a programme of individually colour-banding birds and a territory mapping census conducted over 2-3 weeks in Aug each year, before the beginning of the breeding season (Robertson & Saul 2008). Accurate sex determination of kakerori is important for understanding the population dynamics of this threatened species. Contrary to early descriptions of the species (e.g., Holyoak 1980), which suggested female kakerori have orange plumage and males have grey plumage, a colour-banding study showed that plumage colour is related to age rather than sex (Robertson *et al*. 1993). In both sexes, plumage is orange when the birds are 1-2 years old (1-year old birds have a yellow base to the lower mandible, whereas 2-year-old bird have a completely dark bill), plumage is a mix of orange and grey in the 3rd year, and the definitive grey plumage is attained by both sexes in the 4th year (Robertson *et al*. 1993). With considerably more data from colour-banded birds of known age and putative gender the only variation to this described pattern of plumage change with age

Table 1. Morphometric measurements of kakerori recorded by HAR between 1987 and 2007. The number of individuals is indicated by n, and plumage colour codes (Oy, Ob, M, and G) are as described in Roberston *et al*. (1993).

Measurement / Age (colour)	Female					Male				
	mean	s.d.	n	min	max	mean	s.d.	n	min	max
Bill length (mm)										
1 yo (Oy)	13.24	0.39	109	12.4	14.5	14.11	0.50	148	12.8	15.6
2 yo (Ob)	13.11	0.42	33	12.0	13.9	14.24	0.53	46	13.0	15.8
3 yo (M)	13.13	0.46	8	12.4	13.7	14.27	0.36	14	13.7	14.9
4+ yo (G)	13.11	0.40	20	12.1	13.7	14.12	0.49	38	13.3	15.3
Head and bill length (mm)										
1 yo (Oy)	37.91	0.61	109	36.2	39.5	39.18	0.64	148	37.6	41.1
2 yo (Ob)	37.68	0.70	33	36.5	39.3	39.37	0.65	46	38.4	41.2
3 yo (M)	37.79	0.43	8	37.1	38.4	39.63	0.35	14	39.1	40.1
4+ yo (G)	38.11	0.58	20	36.5	38.8	39.31	0.61	38	38.0	40.3
Tarsus length (mm)										
1 yo (Oy)	24.34	0.53	109	22.5	25.6	25.01	0.53	148	22.8	26.2
2 yo (Ob)	24.25	0.53	33	22.8	25.3	25.24	0.47	46	24.2	26.3
3 yo (M)	24.34	0.36	8	24.0	25.1	25.36	0.37	14	24.6	26.0
4+ yo (G)	24.38	0.50	20	23.2	25.4	25.06	0.54	38	24.1	26.2
Wing length (mm)										
1 yo (Oy)	76.6	1.3	109	74	80	78.6	1.6	148	74	82
2 yo (Ob)	80.2	1.6	33	77	83	83.6	1.4	46	80	86
3 yo (M)	81.6	0.7	8	81	83	84.5	1.3	14	82	87
4+ yo (G)	83.5	1.4	20	81	86	85.0	1.3	38	82	88
Tail length (mm)										
1 yo (Oy)	62.3	1.7	109	56	67	63.3	1.6	148	58	69
2 yo (Ob)	64.5	2.2	33	59	69	65.6	2.0	46	61	69
3 yo (M)	65.3	1.2	8	63	66	66.2	1.4	14	64	69
4+ yo (G)	66.7	1.8	20	62	69	66.9	1.8	38	62	70
Weight (g)										
1 yo (Oy)	19.66	1.15	109	17.2	25.2	21.68	1.20	148	18.3	24.9
2 yo (Ob)	20.66	1.45	33	18.0	24.2	22.74	1.28	46	20.2	27.6
3 yo (M)	20.84	0.71	8	19.9	21.8	22.62	1.09	14	21.4	26.0
4+ yo (G)	21.40	1.16	20	19.1	24.2	23.04	1.25	38	21.1	27.0

is that in the 3rd year, male kakerori are generally greyer than females, and about 10% of such males appear entirely grey in the field, although in the hand or in photographs they are often found to have a few orange-tipped feathers.

Sex determination of kakerori in the field relies mainly on morphometric measurements and behavioural observations. While direct observation of mating behaviour (male on top) is a generally reliable method of sex determination, it requires a

large investment of observer time. Since mating only occurs over 1 to 3 days per pair for each breeding attempt, it is not practical for surveying large numbers of breeding pairs. Morphometric measurements of kakerori, however, do not have sufficient power to discriminate reliably between sexes in all individuals captured, because the sexes have an overlapping distribution for all 6 of the measurements conventionally taken: bill length, head and bill length, tarsus length, wing length, tail length, and weight. The last 3 measurements also vary with age (Robertson *et al*. 1993). The data given by Robertson *et al.* (1993) came from only a small sample of birds (n = 60) and they had to combine 2-year old and 3-year old cohorts. We, therefore, now present data from a much larger sample of birds (n = 416) measured by HAR between 1987 and 2007 (Table 1).

Based on the 6 morphological characters measured by HAR and subsequent pairings and breeding behaviour observed by EKS, about 88% of birds were sexed correctly if it is assumed that females have a bill length of ≤13.6 mm. The accuracy was improved to 91% if it is assumed that all birds with a bill length <13.5 mm are females, and any bird with a bill length of 13.5-13.7 mm is a female if its head and bill length is ≤38.5 mm. Using extra information on tarsus length and other measurements (allowing for the age of the bird), the accuracy of field sexing based on data obtained by HAR has proven to be 90-95% accurate. The problem with sexing kakerori from morphometric measurements alone is that they struggle more than most other passerine species while being held in the hand, making them difficult to measure reliably. This exacerbates differences between researchers in how they take measurements, thus reducing the reliability of sexing from measurements alone.

Advances in molecular genetic techniques have provided alternative methods to address a wide range of problems in ecological studies (e.g. Lambert & Millar 1995). DNA-based avian sexing techniques developed have now been used successfully across a large number of species (Griffiths *et al*. 1998, Fridolfsson & Ellegren 1999). These protocols utilise a size difference in a non-protein coding region (an intron) of the sex-linked gene encoding the chromo-helicase-DNA binding protein on the sex chromosomes (CHD; Ellegren 1996, Griffiths *et al*. 1996). In birds, females are heterogametic (ZW) and males are homogametic (ZZ). Genetic markers, such as the *CHD* gene, on the W chromosome are thus preferred candidates to differentiate between the sexes in birds. Divergent copies of this gene are found on the avian Z and W chromosomes (Griffiths & Korn 1997). Intron size difference between the W chromosome copy (*CHD-W*) and Z chromosome copy (*CHD-Z*) of the *CHD* gene can be detected using the polymerase chain reaction (PCR; Griffiths *et al*. 1998, Fridolfsson & Ellegren 1999). The chief advantage of using PCR-based techniques is their high sensitivity. Hence, they require only small amounts of DNA template and allow the use of minimally invasive biological sample sources such as quills of plucked feathers (Taberlet *et al*. 1999, Bush *et al*. 2005).

The DNA-based sexing methods, however, are not without their own potential problems. For instance, there is a possibility that the W copy of *CHD* may not amplify from some individuals, resulting in mis-scoring of females as males. Hence, these protocols require careful optimisation and testing before applying them for sexing a particular species. It has been recommended that the *CHD* based sexing protocols (Griffiths *et al*. 1998, Fridolfsson & Ellegren 1999), when used alone, should be tested first on individuals of known sex, or that the two protocols should be used in conjunction with one another to cross check results (Dawson *et al*. 2001, Robertson & Gemmell 2006). Unfortunately, in some studies, reliably sexed individuals are unavailable, and only one of the sexing protocols yields discernable *CHD-W* and *CHD-Z* PCR products (e.g. Tokunaga *et al*. 2007). The kakerori provides one such case because our PCR trials using the Griffiths *et al*. (1998) primers did not produce any *CHD* products. We therefore describe testing of DNA-based sexing in conjunction with field sexing of kakerori from morphometrics and behavioural observations to provide a reliable sexing method.

Breast feathers were collected from each of 42 kakerori colour-banded in the 2006 and 2007 field seasons, which included 1 pair of birds with known sex. For each individual, DNA was extracted from the quills of 6 feathers using High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. PCR was performed using the primers 2550F and 2718R as described in Fridolfsson & Ellegren (1999). Briefly, about 5 ng of DNA was used as template for PCR amplification in each 10 μl reaction containing 1 x Ex Taq buffer (TaKaRa Bio., Otsu, Japan), 3 mM $MgCl_2$, 200 μM dNTPs, 2 pmol of each primer, and 0.25 U of Ex Taq polymerase (TaKaRa Bio., Otsu, Japan). The reactions were run in an Eppendorf Mastercycler ep thermocycler using a touchdown regime (Don *et al*. 1991) consisting of an initial denaturation at 94°C for 2 min, followed by 11 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s with a 1°C decrease per cycle, product extension at 72ºC for 40 s, then 35 cycles of (94°C 30 s, 50°C 30 s, 72°C 40 s), and a final extension step of 72°C for 5 min. The PCR products were size separated by electrophoresis on 1% LE agarose gels (Roche Diagnostics, Mannheim, Germany). Gels were run in 1 x TA buffer (40 mM

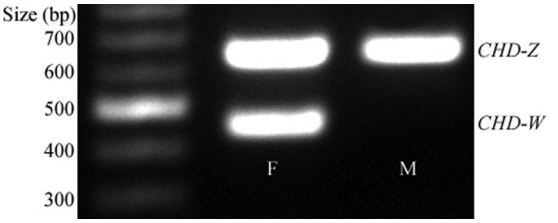

Fig. 1. Gel electrophoresis of kakerori *CHD* PCR products alongside DNA molecular weight marker XIV (Roche Diagnostics, Mannheim, Germany; leftmost lane). Female (ZW; middle lane) shows 2 bands: *CHD-W* and *CHD-Z*. Male (ZZ; rightmost lane) shows 1 band: *CHD-Z*.

Tris, 20 mM acetic acid) with ethidium bromide stain for 60 min at 100 V. DNA fragments on the gels were visualised under UV light. To confirm we had amplified the correct *CHD* target, examples of the kakerori *CHD-W* and *CHD-Z* products were sequenced using the Allan Wilson Centre Genome Service, Massey University, Palmerston North, New Zealand. DNA sequences obtained shared highest homology to *CHD* sequences from other avian species deposited in the DNA Data Bank of Japan (DDBJ; http://www/ddbj/nig/ac/jp).

For morphometric sexing, measurements of bill length, bill plus head length, tarsus length, wing length, tail length and weight were taken by HAR from 34 birds captured in mist-nets in 2006 and 2007, while EKS measured the other 8 birds. Pairing behaviour was recorded during the annual census, or by EKS during the subsequent breeding season.

PCR amplification of kakerori DNA using the Fridolffsson & Ellegren (1999) system resulted in single 651 bp products (*CHD-Z*) for males, a 651 bp (*CHD-Z*) plus a 454 bp products (*CHD-W*) for females (Fig. 1). The DNA sequence of these PCR products showed high similarity to other avian *CHD-W* and *CHD-Z* sequences in DDBJ. The sequences of the kakerori *CHD-W* and *CHD-Z* introns amplified in this study are deposited in DDBJ under accession numbers AB458559 and AB458560. Of the 42 birds screened by DNA-based sexing, 19 were females and 23 were males, and sex was assigned correctly for the pair of known sex birds. The molecular and field sex assignments for 32 (94%) of the 34 birds measured by HAR agreed, but the gender assigned to 2 yearlings was different. One bird (YG-GY) scored as a male by DNA sexing had equivocal morphometric measurements: bill length (13.0 mm) and tail length (62 mm) female-like, wing length (80 mm) and weight (21.8 g) male-like, while head and bill length (38.6 mm) and tarsus length (24.5 mm) were intermediate, but was initially classified as a female by its short bill length. The other bird (YR-GY) scored as a female by DNA sexing had field measurements that were male-like: bill length 13.7 mm, head and bill length 39.4 mm, tarsus length 25 mm, wing length 80 mm, tail length 64 mm, and weight 22.2 g. Unusually, this bird had a grey face and so may possibly have been a 2nd-year bird retaining a yellow base to its bill, which would make the wing and tail lengths more like those of a 2nd-year (Ob) female. The 2nd observer, EKS, who has a pronounced tremor, measured bill and tarsus lengths differently from HAR's methods, and only 3 of the 8 birds he measured, some of them by torchlight, agreed with molecular sexing on bill length alone, but 7 of the 8 agreed on weight alone (based on the criteria in Robertson *et al*. 1993).

Overall, there was a 94% agreement between the morphometric and molecular sexing methods. Errors in the field or the laboratory, or both, could be the cause of the 6% disagreement. Our study suggests that both methods can be dependable for sexing kakerori. The morphometric method provides instant results in the field, but may be prone to errors when different researchers of varying ability and experience take the measurements, and also returns equivocal measurements for some individuals when year cohort is uncertain. The DNA-based method is a valuable additional tool for discriminating sexes in such circumstances and when individuals have equivocal morphometric measurements, but it requires processing time in a laboratory setting. While DNA-based methods are gaining popularity in avian sexing, a verified morphometric sexing method has definite practical advantages for field work. For accurate sexing of kakerori, we recommend using both morphometric and DNA sexing techniques, where possible. Rather than being an "ultimate tool" for sexing birds, we suggest that, in many cases, the real value of molecular sexing may be its use for re-examining and improving morphometric sex assignment methods, and highlighting uncertainties and errors in field sexing.

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