Notornis, 2007, Vol. 54: 38-41
0029-4470 © The Ornithological Society of New Zealand, Inc.

SHORT NOTE

Morphological sexing of Forbes' parakeet (*Cyanoramphus forbesi*) validated by molecular data

CHIHIRO TOKUNAGA
WEN-JIUN LAM
Institute for Molecular Systematics, School of Biological Sciences, Victoria University of Wellington, P.O. Box 600, Wellington, New Zealand

DENISE FASTIER
Chatham Islands Area Office, Department of Conservation, P.O. Box 114, Chatham Islands, New Zealand

HILARY AIKMAN
Wellington Conservancy, Department of Conservation, P.O. Box 5086, Wellington, New Zealand

CHI-HANG CHAN*
chihang_chan@yahoo.co.nz
GEOFFREY K. CHAMBERS
Institute for Molecular Systematics, School of Biological Sciences, Victoria University of Wellington, P.O. Box 600, Wellington, New Zealand

Forbes' parakeet (*Cyanoramphus forbesi*) is an endangered taxon now endemic to Mangere I and Little Mangere I in the Chatham Is group, 500 km east of New Zealand. This taxon exists now as a single mixed population consisting of Forbes' parakeets, Chatham I red-crowned parakeets (*C. novaezelandiae chathamensis*) and their hybrids (Taylor 1975; Nixon 1982; Chan *et al.* 2006). Increased attention on the conservation of Forbes' parakeets followed from the presentation of allozyme and mitochondrial DNA control region genetic evidence which suggested that Forbes' parakeet should be elevated from a subspecies of yellow-crowned parakeet (*C. auriceps forbesi*) to full species status (Triggs & Daugherty 1996; Boon *et al*. 2000).

Little is known about mating preferences of the 2 taxa of parakeet on Mangere I or of their hybrids. A study of the mitochondrial DNA control region (Ballantyne *et al.* 2004) suggested that genetic introgression is bi-directional, and involves females and males of both parent species and hybrids. Field studies of mating behaviour and preferences, and breeding ecology, require the ability to determine the sex of birds accurately. *Cyanoramphus* parakeets do not display overt sexual dimorphism, so there are very few morphological differences between the sexes in adult or young Forbes' parakeets (Nixon 1982).

Recent advances in molecular biology have provided a wide range of DNA-based techniques to study ecological and evolutionary problems (Arnheim *et al*. 1990; Ellegren 1992; Burke 1994; Lambert 1995; Lambert & Millar 1995; Chambers & MacAvoy 1999; Sunnucks 2000). Female birds are the heterogametic sex (ZW); males are homogametic (ZZ). The difference offers the possibility of using W-linked genes as markers to differentiate between the sexes, and to verify any existing sexing system based on morphometrics. A commonly-used W-linked locus for sexing birds is the gene encoding chromo-helicase-DNA binding protein (CHD; Ellegren 1996; Griffiths *et al.* 1996). There is also a divergent copy of the *CHD* gene on the Z chromosome (Griffiths & Korn 1997), and 2 protocols that take advantage of differences in intron size between the W (*CHD-W*) and Z (*CHD-Z*) copies of *CHD* gene are used frequently as molecular markers to sex birds (Griffiths *et al.* 1998; Fridolfsson & Ellegren 1999). These protocols use the polymerase chain reaction (PCR), which requires only a small amount of DNA template, allowing use of non-invasive samples such as shed

Received 24 April 2006; accepted 10 November 2006
*Author for correspondence

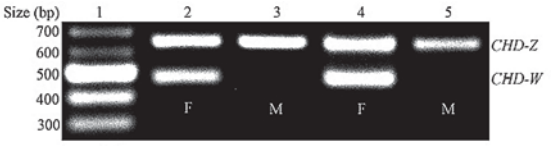

Fig. 1 Gel electrophoresis of *CHD* PCR products alongside DNA molecular weight marker XIV (Roche Diagnostics; lane 1). PCR amplification from Forbes' parakeets (*Cyanoramphus forbesi*) (lanes 2 and 3) and Chatham Is red-crowned parakeets (*Cyanoramphus novaezelandiae*) (lanes 4 and 5) showed 2 bands in females (ZW; lanes 2 and 4), and 1 band in males (ZZ; lanes 3 and 5).

feathers, and hence do not stress the birds involved (Taberlet *et al.* 1999; Bush *et al*. 2005). With such non-invasive sampling, it is, however, essential to know that the material sampled came from the individual to be sexed.

The published protocols do require careful optimisation and quality assurance testing before they can be extended to sex a particular species accurately (Dawson *et al.* 2001; Robertson & Gemmell 2006). We applied this molecular sexing technique to the mixed population of parakeets on Mangere I, Chatham Is, and have developed a protocol that allows adults and chicks of both species and of their hybrids to be sexed accurately for future ecological studies. We have also compared the results of applying the molecular method with those from conventional morphological measurements of bird characteristics, to develop a reliable sexing system for use in the field.

Blood and feather samples were taken from banded individuals on Mangere I during the southern summer field seasons from 1999 to 2001 inclusive. We measured bill length, bill width, wing length, tail length, tarsus length, and body weight as described in Nixon (1982) and Greene (2000). In addition, blood samples were taken from Chatham Is red-crowned parakeets on South-East I (Rangatira).

Total DNA was extracted from 3 μl aliquots of blood samples using a phenol/chloroform method (Sambrook et al. 1989), or from the tips of 1-7 feathers with DNeasy Tissue Kit (Qiagen, Hilden, Germany). About 5 ng of extracted DNA was used as template for PCR amplification in each 10 μl reaction containing 1 × Ex Taq buffer (TaKaRa Bio., Otsu, Japan), 2 mM $MgCl_2$, 200 μM dNTPs, 2 pmol each of primers 2550F and 2718R (Fridolfsson & Ellegren 1999), and 0.25 U of Ex Taq polymerase (TaKaRa Bio., Otsu, Japan). Thermal cycling was performed on an Eppendorf Mastercycler ep thermalcycler: DNA templates in the reactions were denatured initially at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 47°C for 30 s; and product extension at 72°C for 1 min, before a final prolonged extension step of 72°C for 5 min.

The PCR products were separated electrophoretically for 40 min at 100 V on 1% LE agarose gels (Roche Diagnostics, Mannheim, Germany) run in 1× TA buffer (40 mM Tris, 20 mM acetic acid). The gels were stained with ethidium bromide solution (Sambrook *et al*. 1989) and DNA fragments were visualised under UV light. To confirm we had amplified the correct *CHD* target, we also sequenced 1 example of *CHD-W* and *CHD-Z* PCR products on an Applied Biosystems ABI Prism 377 automated sequencer and compared our sequences to those published on the DDBJ online database (http://www.ddbj.nig.ac.jp).

We performed trials with both Griffiths *et al*. (1998) and Fridolfsson & Ellegren (1999) general molecular sexing protocols and found the later protocol more efficient for sexing parakeets on Mangere I. Using the modified Fridolffsson & Ellegren (1999) system, the *CHD-W* PCR product was *c*.450 bp and the *CHD-Z* product *c*.650 bp in Forbes' parakeets, Chatham Is red-crowned parakeets, and in their hybrids. Thus, male birds (ZZ) showed a single electrophoretic band whereas females (ZW) showed 2 bands that could be clearly differentiated on an agarose gel image (Fig. 1). The DNA sequences of the PCR products showed high levels of homology to published *CHD-W* and *CHD-Z* sequences from other avian species on DDBJ (DNA sequences of the *CHD-W* and *CHD-Z* introns amplified are deposited in DDBJ under accession numbers AB247266-AB247269). The protocol worked well for Forbes' parakeets, Chatham Is red-crowned parakeets and hybrids. whereas the Griffiths *et al*. (1998) method did not result in electrophoretically distinguishable W and Z products in the parakeets we examined.

The PCR-based molecular sexing method developed here is very robust and requires only a small amount of DNA as a starting template. We had success with the protocol using DNA extracted from a single contour feather. The quality of the results appears to depend on the quality of the sample. Generally, feathers with even small amounts of skin tissue attached to their tips consistently provided sufficient good quality template DNA. Feather samples collected on Mangere I for this study were stored dry at room temperature in clean paper envelopes for shipping (up to 3 month's duration), and they appeared to give a good success rate (>90%). The molecular sexing protocol described here involves a minimal, easily portable, set of laboratory equipment, and therefore can be performed in a field station with an adequate electricity supply.

While optimising this protocol, we observed preferential amplification of the larger *CHD-Z* product over the smaller *CHD-W* product. When the original Fridolfsson & Ellegren (1999) general protocol was used without modification, only the

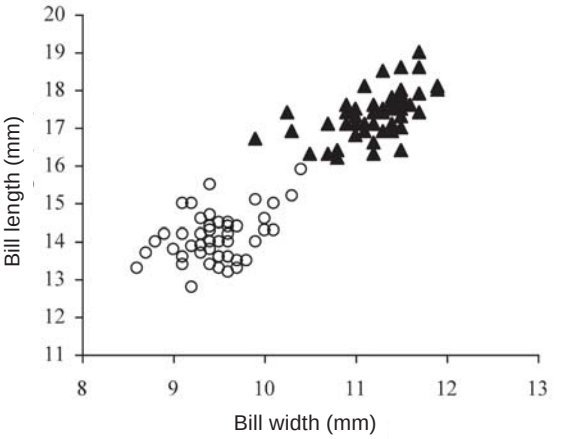

Fig. 2 Relationships between bill width and bill length, and sex in adult *Cyanoramphus* parakeets on Mangere I, Chatham Is; ○,females; ▲, males.

CHD-Z product could be amplified. Thus, caution should always be exercised when sample quality is poor because there is a slight risk that the *CHD-W* product will not be amplified, resulting in mis-scoring females as males. To ensure accuracy of assignment, it is also advisable to repeat the PCR amplification (varying the amount of input DNA) on poor quality templates if only the *CHD-Z* product is amplified.

In total, we sexed 105 adult parakeets and 50 nestlings. The age or developmental stage of the birds did not affect the success of the sexing protocol. We also re-examined the correlations between various morphological variables with the sex of adult birds. In general, we found that there were few sex specific differences in most morphological characters (Table 1). Nixon (1982) reported significant differences in bill size between sexes, but our study showed that bill size alone was not completely reliable for sexing *Cyanoramphus* parakeets because female and male bills overlap in size (Fig. 2).

There were no differences in bill dimensions between species. Bill lengths and widths were bimodally distributed, which suggested, however, that some individuals could be sexed by their bill size (Nixon 1982). Our molecular data allowed individuals (Fig. 2) to be classified by sex, and showed that females and males did cluster separately when bill width was plotted against bill length. Thus, most adult parakeets on Mangere I could be sexed with reasonable confidence in the field by a combination of morphological measurements (Table 1), mainly bill sizes (females: bill length ≤ 15.3 mm, bill width < 9.9 mm; males: bill length ≥ 16.2 mm; bill width > 10.4 mm). However, adult bill widths of the sexes overlapped and the maximum female bill length (15.9 mm) approached the minimum male bill length (16.2 mm; Table 1), which suggested that the molecular method was the better means to sex adult *Cyanoramphus* parakeets. It is important to be able to sex the unfledged chicks of *Cyanoramphus* parakeets because it is convenient to band them at this stage to facilitate later observational studies. Their beaks are still growing, so the molecular method is the only means of sexing chicks and chicks and fledglings.

Table 1 Dimensions (mm) and weight (g) of sexes of adult *Cyanoramphus* parakeets on Mangere I, Chatham Is. Range, mean ± SD.

Dimension	Female	Male
Bill length	12.8-15.9	16.2-19.0
	14.1 ± 0.6	17.4±0.6
Bill width	8.6-10.4	9.9-11.9
	9.5 ± 0.4	11.2±0.4
Wing length	116.5-138.5	127.0-148.0
	127.0 ±5.0	135.6±4.2
Tail length	128.0-161.0	115.0-173.0
	145.1±8.2	153.5±10.3
Tarsus length	19.2-21.8	20.4-23.0 21.8±0.7
	20.35 ± 0.6	
Weight	57-82	71-99
	70 ± 6	87±6

The ability to accurately sex adult parakeets and their chicks will provide new opportunities for studies on the breeding behaviour of parakeets. Understanding the basis for mate choice and breeding success in relation to hybridisation will provide valuable information for the development of management plans amid at reducing or preventing further hybridisation between Forbes' and Chatham Is red-crowned parakeets.

ACKNOWLEDGEMENTS

This study was supported by the New Zealand Department of Conservation (DoC) and Victoria University of Wellington. We thank Tertia Thurley and other DoC field staff for collecting samples, and Deborah Dawson (University of Sheffield, U.K.) for sharing information related to DNA-based sexing protocols.

LITERATURE CITED

Arnheim, N.; White, T.; Rainey, W.E. 1990. Application of PCR: Organismal and population biology. *BioScience 40*: 174-182.

Ballantyne, K.N.; Chan, C-H.; Chambers, G.K. 2004. A PCR-RFLP based method for assigning mitochondrial control region haplogroups in hybridizing Chatham Islands *Cyanoramphus* parakeets. *New Zealand natural sciences 29*: 33-38.

Boon, W.M.; Kearvell, J.C.; Daugherty, C.H.; Chambers, G.K. 2000. Molecular systematics of New Zealand *Cyanoramphus* parakeets: Conservation of orange-fronted and Forbes' parakeets. *Bird conservation international 10*: 211-239.

Burke, T. 1994. Spots before the eyes: Molecular ecology. *Trends in ecology and evolution 9:* 355-357.

Bush, K.L.; Vinsky, M.D.; Aldridge, C.L.; Paszkowski, C.A. 2005. A comparison of sample types varying in invasiveness for use in DNA sex determination in an endangered population of greater sage-grouse (*Centrocercus uropihasianus*). *Conservation genetics 6:* 867-870.

Chambers, G.K.; MacAvoy, E.S. 1999. *Molecular genetic analysis of hybridisation*. *Science for conservation no. 105*. Wellington, New Zealand Department of Conservation.

Chan, C-H.; Ballantyne, K.N.; Aikman, H.; Fastier, D.; Daugherty, C.H.; Chambers, G.K. 2006. Genetic analysis of interspecific hybridisation in the world's only Forbes' parakeet (*Cyanoramphus forbesi*) natural population. *Conservation genetics 7*: 493-506.

Dawson, D.A.; Darby, S.; Hunter, F.M.; Krupa, A.P.; Jones, I.L.; Burke, T. 2001. A critique of avian *CHD*-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets. *Molecular ecology notes 1*: 201-204.

Ellegren, H. 1992. Polymerase-Chain-Reaction (PCR) analysis of microsatellites – a new approach to studies of genetic relationships in birds. *Auk 109*: 886-895.

Ellegren, H. 1996. First gene on the avian W chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *Proceedings of the Royal. Society of London B 263*: 1635-1641.

Fridolfsson, A-K.; Ellegren, H. 1999. A simple and universal method for molecular sexing of non-ratite birds. *Journal of avian biology 30*: 116-121.

Greene, T.C. 2000. Forbes' parakeet (*Cyanoramphus forbesi*) population on Mangere Island, Chatham Islands. *Conservation advisory science notes no. 319*. Wellington, New Zealand Department of Conservation.

Griffiths, R.; Dann, S.; Dijkstra, C. 1996. Sex identification in birds using two CHD genes. *Proceedings of the Royal. Society of London B 263*: 1251-1256.

Griffiths, R.; Korn, R.M. 1997. A *CHD1* gene is Z chromosome linked in the chicken *Gallus domesticus*. *Gene 197*: 225-229.

Griffiths, R.; Double, M.C.; Orr, K.; Dawson, R.J.G. 1998. A DNA test to sex most birds. *Molecular ecology 7*: 1071-1075.

Lambert, D.M. 1995. The new science of molecular ecology. *New Zealand journal of ecology 19*: 93-96.

Lambert, D.M.; Millar, C.D. 1995. DNA science and conservation. *Pacific conservation biology 2*: 21-38.

Nixon, A.J. 1982. Aspects of the ecology and morphology of *Cyanoramphus* parakeets and hybrids from Mangere Island, Chatham Islands. Unpubl. MSc thesis, Victoria University of Wellington, Wellington, New Zealand.

Robertson, B.C.; Gemmell, N.J. 2006. PCR-based sexing in conservation biology: wrong answers from an accurate methodology? *Conservation genetics 7: 267-271.*

Sambrook, J.; Fritsch, E.F.; Maniatis, T. 1989. *Molecular cloning: A laboratory manual*. *2nd ed*.. New York, Cold Spring Harbor Laboratory Press.

Sunnucks, P. 2000. Efficient genetic markers for population biology. *Trends in ecology and evolution 15*: 199-203.

Taberlet, P.; Waits, L.P.; Luikart, G. 1999. Noninvasive genetic sampling: Look before you leap. *Trends in ecology and evolution 14*: 323-327.

Taylor, R.H. 1975. Some ideas on speciation in New Zealand parakeets. *Notornis 22*: 110-121.

Triggs, S.J.; Daugherty, C.H. 1996. Conservation and genetics of New Zealand parakeets. *Bird conservation international 6*: 89-101.

Keywords Forbes' parakeet; *Cyanoramphus forbesi*; red-crowned parakeet; *Cyanoramphus novaezelandiae*; sexing; PCR; *CHD*; hybridisation; morphometrics