

A PCR-RFLP based method for assigning mitochondrial control region haplogroups in hybridizing Chatham Islands *Cyanoramphus* parakeets

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(Received 28 August 2004, revised and accepted 16 November 2004)

Abstract

The two endemic Chatham Islands parakeet species, *Cyanoramphus forbesi* (Forbes' parakeet) and *C. novaezealandiae chathamensis*, (Chatham Islands Red-crowned parakeet) have had a long history of hybridisation which has increased recently as a result of habitat modification. Previous genetic research showed the introgression of ancestral Red-crowned parakeet mtDNA into Forbes' parakeet, resulting in four distinct mitochondrial haplogroups spread across the two parent species and their hybrids. A PCR-RFLP assay was designed to determine individuals' haplogroup membership and thus calculate the extent of hybridisation between the two species. It was found that the majority of individuals belong to the ancestral Forbes' parakeet haplogroup, with a significant number of Forbes' parakeets having the ancestral Red-crowned parakeet mitochondrial types. A small number of individuals from both species had mtDNA of the other species, but this was not reflected in the plumage of the parakeets, suggesting the presence of cryptic hybrids in the Chatham Islands parakeet population.

Keywords: mitochondrial DNA control region - *Cyanoramphus* - PCR-RFLP - hybridisation.

Introduction

Forbes' parakeet (*Cyanoramphus forbesi*) is an endangered New Zealand parrot, currently found only on Mangere and Little Mangere Islands within the Chatham Group. Due to widespread forest clearance on Mangere Island in the past century, population levels

declined rapidly until in the 1960's there was an estimated 30 individuals remaining (Taylor 1985). It was suggested that the loss of habitat and low population numbers led to extensive hybridisation with the closely related Chatham Islands Red-crowned parakeet, *C. novaezealandiae chathamensis*, a previously allopatric

species which had colonised the modified environment of Mangere Island (Nixon 1982).

Genetic research using the mitochondrial DNA (mtDNA) control region has discovered the existence of four distinct mitochondrial haplogroups within Forbes' and Chatham Island Red-crowned parakeets (Boon *et al.* 2001). Haplogroups 1 and 2 represent relatively ancient hybridisation between these two parakeet species, leading to the introgression of ancestral Red-crowned parakeet mtDNA into the Forbes' parakeet population. Haplogroup 3 represents the ancestral lineage of Forbes' parakeet and haplogroup 4 is limited to the Chatham Islands Red-crowned parakeet. The study suggested that there has been a prolonged history of hybridisation within the two Chatham Islands parakeet species, leading to the existence of Forbes' parakeets with the Red-crowned parakeet mitochondrial haplogroup.

There are a wide variety of crown plumage variants within the Mangere Island parakeet population – pure yellow crowned parakeets with red frontal band (Forbes' parakeets), ranging through orange or yellow-red crowns to red-crowned parakeets (*C. n. chathamensis*). Although the intermediate forms were assumed to be hybrids, previous genetic studies (Boon *et al.* 2001) showed that some yellow-crowned Forbes' parakeets contain Red-crowned parakeet mtDNA. Here we present a PCR-RFLP based assay for assigning mtDNA haplogroups in Chatham Islands *Cyanoramphus* parakeets. This method removes the need for DNA sequencing and allows a

large number of samples to be screened in a short period of time at a reduced cost. Similar techniques have been developed to successfully distinguish between mtDNA variants in other species (for example, Palo & Merilä 2003).

Methods and Materials

The PCR-RFLP assay was designed using bioinformatics analysis of mitochondrial control region sequences, produced by Boon *et al.* (2001; DDBJ accession numbers AB179749, AB179750, AF218740, AF218744), with the DNASTar 4.0 software (<http://www.DNASTar.com>). The sequences were screened for diagnostic changes, and restriction enzymes were selected which had recognition sites in only one of the four haplogroups. Feather and blood samples were analysed from 212 Forbes' and Red-crowned parakeets. DNA was extracted using a phenol/chloroform extraction method (Sambrook *et al.* 1989) or DNeasy extraction kit (Qiagen). The 2.5kb mitochondrial control region was amplified using a nested PCR protocol. Initially, the primers L16518 (5'-GACGGGAATAAACAAAAACCACCAACA) and H1800 (5'-CCCCCGTTTGTGCTCGTAGTTCTC; Boon *et al.* 2001) were used to amplify a large segment encompassing the entire control region. PCR reactions were performed with 20ng genomic DNA, 0.2mM dNTPs, 1x Expand High Fidelity PCR Buffer (Roche), 1.5mM MgCl₂, 0.5mM of each primer and 0.05U Expand High Fidelity DNA polymerase (Roche). The cycling profile, run on a Perkin-Elmer 480 thermocycler, consisted of 95°C for 3 min, followed by 35 cycles of 95°C

for 15 sec, 55°C for 30 sec and 68°C for 2 min, with a cumulative increase of extension time of 20 sec per cycle for the last 25 cycles, followed by a final extension step at 68°C for 7 min. The second nested PCR was carried out under the same PCR conditions using primers L90-110 (5'-AACTTCACG CCCTCGGATAGAATA) and H1529 (5'-TGGCTGGCACAAGATTT ACCG; Boon *et al.* 2001), with 40ng of PCR product from the first PCR as template. This resulted in the amplification of a 1.6kb section of the mitochondrial DNA control region. The PCR products from the second PCR were purified with High Pure PCR Products Purification Kit (Roche) and digested with the restriction enzymes selected. Each restriction digest was performed at 37°C for 2 hours in a 20ml total reaction volume containing the 1.6kb mitochondrial DNA control region PCR product and 10U of restriction enzyme in the appropriate 1x SURE/Cut Buffer (Roche) as recommended by the manufacturer. The enzyme *HaeIII* was used to give an initial indication of haplogroup membership. Digestion with *HaeIII* gave identical fragment patterns of 24 (not visible on a 1.7% agarose gel), 97, 192, 380, 393 and 535bp for haplogroups 2 and 4, while haplogroup 1 gave fragments of 97, 371, 381 and 727bp, and haplogroup 3 of 69, 97, 162, 191, 330 and 751bp (Figure 1; the 380/393 and 371/381bp bands in haplogroups 1, 2 and 4 appear as single bands on a 1.7% agarose gel). Three other enzymes were then used to confirm haplogroup membership. Haplogroup 3 produced fragments of 355bp and 1245bp when digested with *Clal*,

which did not cut DNA obtained from any of the other haplogroups. In contrast, *HindIII* cut DNA amplified from haplogroups 1 and 2 to give 398bp and 1202bp fragments, while *RcaI* cut only haplogroup 2 PCR products producing fragments of 111bp and 1489bp. Table 1 summarises the diagnostic restriction digestion system for the four haplogroups. Digest fragments were analysed on 1.7% agarose gels (Agarose MS, Roche). Random subsets of samples were sequenced on an ABI Prism 377 sequencer (Applied Biosystems) using the nested primers, confirming the accuracy of the PCR-RFLP assay.

Results

The PCR-RFLP assay clearly distinguished between the four haplogroups, allowing the assignment of each individual to a haplogroup. A total of 215 birds were tested from Mangere Island, although 41 were excluded from analysis due to lack of phenotypic data. On Mangere Island, 61% of individuals tested belong to the ancestral Forbes' parakeet haplogroup 3, 16% and 11% belong to haplogroups 1 and 2 respectively and 12% to the Red-crowned parakeet haplogroup.

Table 1. PCR-RFLP system for Forbes' parakeet haplogroups. + indicates restriction enzyme cuts the PCR products; - indicates no digestion; * indicates haplogroup can be identified by sizes of digestion products.

Haplogroup	<i>HaeIII</i>	<i>Clal</i>	<i>HindIII</i>	<i>RcaI</i>
1	+	-	+	-
2	+	-	+	+
3	+	+	-	-
4	+	-	-	-

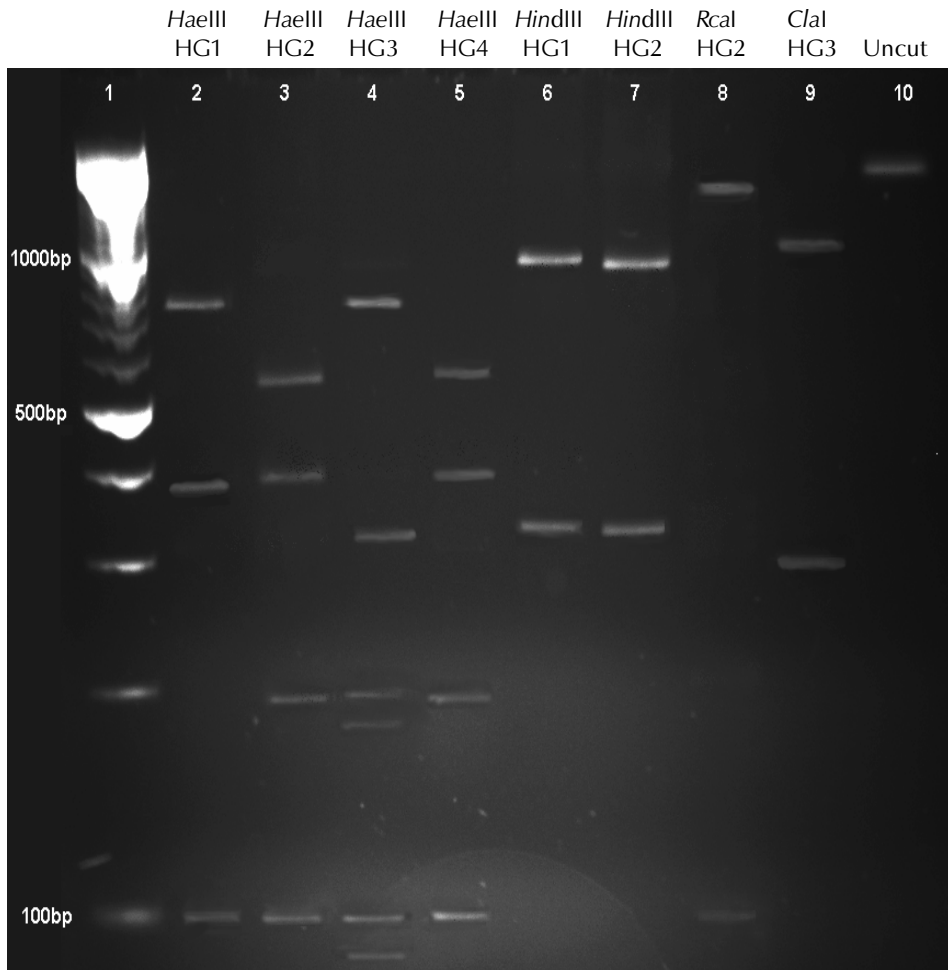


Figure 1. Differing restriction fragment patterns of the 1600bp mitochondrial control region in *C. forbesi* and *C. novaezealandiae chathamensis*. The fragments were run together with a Molecular Marker XIV (Roche, Lane 1) at 40V for 15 hours on a 1.7% agarose gel. Lanes: 2 – *Haelll* digest of Haplogroup 1 PCR product (HG1); 3 – *Haelll* digest HG2; 4 – *Haelll* digest HG3; 5 – *Haelll* digest HG4; 6 – *Hindlll* digest HG1; 7 – *Hindlll* digest HG2; 8 – *Rcal* digest HG2; 9 – *Clal* digest HG3; 10 – undigested PCR product.

Morphological examinations identified 72% of the population as Forbes' parakeets, 7% as Red-crowned parakeets and 21% as hybrids (having plumage intermediate of the two parent species). Six phenotypic Forbes' parakeets contained Red-crowned parakeet mtDNA, and two phenotypic Red-crowned parakeets contained haplogroup 2 mtDNA. The majority

(70%) of phenotypic hybrids had haplogroup 3 mtDNA, 8% had haplogroup 1 or 2 mtDNA, and 14% had Red-crowned parakeet mtDNA (Table 2).

We also examined 34 samples from South-East Island (Rangatira), about 13km from Mangere Island, which is inhabited only by a population of Chatham Islands Red-crowned

Table 2. Results from PCR-RFLP assay for Mangere Island parakeets, separated into mtDNA haplogroup and phenotype [sample size (percentage of mtDNA haplogroup) / (percentage of phenotype)].

MtDNA Haplotype	Forbes'	Phenotype Hybrid	Red-Crowned
1	24 (89%) / (19%)	3 (11%) / (8%)	0
2	15 (75%) / (12%)	3 (15%) / (8%)	2 (10%) / (17%)
3	80 (75%) / (64%)	26 (25%) / (70%)	0
4	6 (29%) / (5%)	5 (24%) / (14%)	10 (47%) / (83%)

parakeets. Among the samples tested, 12% were found to have haplogroup 2 mtDNA, with the remainder having haplogroup 4 mtDNA.

Discussion

The existence of low numbers of Forbes' parakeets with Red-crowned parakeet mtDNA and vice versa suggests that hybridisation is currently occurring at relatively low levels. About 5% of birds with the Forbes' parakeet phenotype have Red-crowned mtDNA and 31% of Forbes' parakeets contain ancestral Red-crowned mtDNA (haplogroups 1 and 2). It would appear that plumage characteristics cannot be used as a reliable marker to identify hybrids in the field, given that there are a significant number of Forbes' parakeets which have mtDNA from Red-crowned parakeets (haplogroup 4 and the ancestral 1 and 2 groups). A number of phenotypic hybrids had haplogroup 3 mtDNA, which shows that their maternal ancestors were Forbes' parakeets. However, this does not rule out hybridisation with male Red-crowned parakeets.

We are currently developing a system using microsatellite markers for testing the true hybrid status of these

birds, and to determine the correlation between genetic hybrid status and plumage characteristics. The assignment of a number of South-East Islands Red-crowned parakeets to haplogroup 2 suggests there may be previously unnoticed introgression of Forbes' parakeet genes into the Red-crowned parakeet species, or the ancestral haplogroup has been retained in a small number of individuals.

The PCR-RFLP method we developed allowed us to successfully assign parakeets from the Chatham Islands to the different haplogroups. This study supported earlier observations by Boon *et al.* (2001) on the lack of correlation between phenotypic characters and mtDNA haplogroups in Mangere Island parakeets. Genetic testing is needed to reliably identify *Cyanoramphus* hybrids.

Acknowledgements

We thank Hilary Aikman and Colin Miskelly of the NZ Department of Conservation for providing samples and funding, Elizabeth MacAvoy, James Sainsbury, and Wee-Ming Boon for advice about laboratory techniques and data analysis.

References

- Boon, W.M., Kearvell, J.C., Daugherty, C.H. & Chambers, G.K. (2001). Molecular systematics and conservation of Kakariki (*Cyanoramphus* spp.). *Science for Conservation* 176. Department of Conservation, New Zealand.
- Nixon, A.J. (1982). Aspects of the ecology and morphology of *Cyanoramphus* parakeets and hybrids from Mangere Island, Chatham Islands. Unpublished M.Sc. Thesis, Victoria University of Wellington, New Zealand.
- Palo, J. & Merilä, J. (2003). A simple RFLP method for identification of two Ranid frogs. *Conservation Genetics* 4: 801-803.
- Sambrook, J., Fritsch, E.J. & Maniatis, T., (1989). *Molecular Cloning: A Laboratory Manual* (2nd edition). Cold Spring Harbour Laboratory Press, New York.
- Taylor, R.H. (1985). Status, habits and conservation of *Cyanoramphus* parakeets in the New Zealand region. In *Conservation of Island Birds* (ed P.J. Moore), ICBP Technical Publications 3, pp. 195-211. Moore Cambridge Press.