Genetic analysis of Forbes' parakeet *(Cyanoramphus forbesi)*

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Summary

The taxonomic status of Forbes' parakeet has remained unresolved for several decades. With the advent of modern molecular biological techniques, it has become possible to augment traditional taxonomic practices (i.e. those based on morphology) with DNA data analysis. The conservation status of Forbes' parakeet (presently listed by as "critically threatened") warrants urgent research into its genetics. In this report, DNA sequencing techniques have been used to analyse a hypervariable mitochondrial DNA (mtDNA) gene target, the control region. Our data provide conservation managers with a new and more complete phylogeny of Forbes' parakeet and its relationship to other New Zealand *Cyanoramphus* parakeets. Our data support a previous claim based on molecular data that Forbes' parakeet fulfils the criteria for specific status.

1. Introduction

The taxonomy of Forbes' parakeet is enigmatic. It was originally described by Rothschild (1893) as a distinct species (C. *forbesi)* but was later relegated to subspecies of the yellow-crowned parakeet (C. *auriceps forbesi)* by Oliver (1930) without any apparent justification. Based on allozyme data, Triggs & Daugherty (1996) found that Forbes' parakeet is genetically very distinct (Nei's D = 0.05) from yellow-crowned parakeet and should again be considered a separate species. Recent work in the Institute for Molecular Systematics (IMS) laboratories at Victoria University of Wellington (VU W) by W. M. Boon (unpublished data) has added further support for this view. DNA analysis of five Forbes' parakeet individuals between May 1996 and March 1999 supports raising of Forbes' parakeet to the rank of a full species. Following the Forbes' Parakeet Technical Meeting (March 1999, DOC, Wellington Conservancy), it was decided that it would be prudent to analyse a larger number of Forbes' parakeet specimens in order to better understand the complex phylogenetic pattern that has begun to emerge. IMS was commissioned by the New Zealand Department of Conservation to extend the phylogenetic analysis by a further five individuals (see Appendix 1 for full description of the analytical services requested).

2. Methods

DNA was extracted from six blood samples of Forbes' parakeet present in the National Frozen Tissue collection held by the IMS at VUW using a phenol-chloroform/Proteinase K protocol (Leeton et al. 1993). Mitochondrial control region targets were then amplified by polymerase chain reaction (PCR),

and the double stranded (ds) DNA products were purified by electrophoresis in 1 % low-melting point agarose gels (FMC Bioproducts). The 2.5 kb product bands were excised from the gel and extracted using DNA purification kits (Biorad). The concentrations of the dsDNA amplification products were estimated visually following electrophoresis with the high DNA mass standard (Life Technologies). Cycle DNA sequencing was performed on the dsDNA products in a Perkin Elmer model 2400 thermal cycler and ABI model 377 Prism automated DNA sequencer (Perkin-Elmer). Consensus DNA sequences for each individual were aligned with Lasergene's MegAlign program (Anon 1997) using the Clustal algorithm and exported to PAUP* 4.02b (Swofford 1998) for phylogenetic analysis. A model-fitting process was carried out to determine the most appropriate mutational substitution model for phylogenetic inference. A maximum likelihood analysis was then carried out based on the Hasegawa-Kishino-Yano 85 (Hasegawa et al. 1985) model with gamma approximation of variable sites ($\alpha =$ 0.239). Bootstrap resampling (Felsenstein 1985) was also carried out (500 replicates) to provide an assessment of statistical support for each bifurcating node. The individuals analysed are listed below:

Forbes' parakeet specimens analysed prior to April 1999:

PMC26, CD1811, CD1814, CD1815, CD 1821

Forbes' parakeet specimens analysed May/June 1999:

CD 1810, CD 1816, CD 1817 CD 1818, CD1819, CD1920

3.   Results and discussion

Six additional Forbes' parakeet specimens from Mangere Island (Chatham Islands) were analysed for the mitochondrial DNA target region specified, bringing the total number of Forbes' parakeet control region sequences in our database to eleven. The new data are in accord with our earlier findings reported by W. M. Boon at the Forbes' parakeet Technical Meeting (1999). As reported previously, Forbes' parakeet individuals all belong to one of three distinct mitochondrial haplotype groups (see Figures 1 and 2). Haplotype Group 1 is closely related to Chatham Island red-crowned parakeets suggesting that these birds may be the result of recent hybridisation, where the female ancestor(s) was/were a red-crowned parakeet. Haplotype Group 2 is basal within the red-crowned parakeet clade and this may represent a much more ancient introgresssion event where hybridisation was again directional (red-crowned parakeet female x Forbes' parakeet male) or an artefact due to mitochondrial lineage sorting. Haplotype Group 3 is highly divergent with respect to all other New Zealand species in the genus *Cyanoramphus.* Thus the latter haplotype group may represent the true matrilineal line of Forbes' parakeet. Figure 1 is an alignment of variable sites for all Forbes' parakeet mtDNA control region sequences. The DNA sequence obtained from specimen CD1816 is used as a reference in Figure 1; dashes indicate insertion/

deletion events and dots indicate identical nucleotides to the reference sequence (shown in red). Figure 2 is a mtDNA control region sequence phylogram containing data from all eleven Forbes' parakeets plus Chatham Island red-crowned parakeet, orange-fronted parakeet, Antipodes Island green parakeet and New Caledonia red-crowned parakeet. These data will be reported in full in a final report to fulfil our contract (number 2119) with the New Zealand Department of Conservation for "Determination of the specific status of the orange-fronted parakeet".

4.   References

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Appendix 1

Department of Conservation Order for External Unprogrammed Science Advice / Services

Analysis of mtDNA samples from 5 Forbes' parakeet has indicated that the population contains two different maternal lines: ancient stock that are the most distinct *Cyanorampbus* parakeet in the New Zealand region; and a more recent derivation of red-crowned stock (but not due to recent hybridisation). This is a surprising result for such a small population that has gone through a severe recent population bottleneck, and it is possible that this unusual result is artefactual. We need further corraboration of a) the existence of ancient mtDNA and b) the presence of 2 independently derived mtDNA lines within the Forbes' parakeet population to allow correct interpretation of the phylogeny and taxonomy of this endangered parakeet. Five further samples of Forbes' parakeet blood are known to exist in the VUW ultrafreezer (based on register records).

This bid is for DNA sequencing of mtDNA extracted and amplified from these samples to allow comparison with sequences obtained from a variety of *Cyanorampbus* taxa as part of Wee Ming Boon's PhD research.

Question for which advice/service required

Produce a phylogenetic tree of *Cyanorampbus* parakeets showing the locations of Forbes' parakeets based on partial sequencing of mtDNA of ten independent blood samples (five of which have already been sequenced) and interpretation of the likely cause(s) of such a branching pattern and its taxonomic implications.
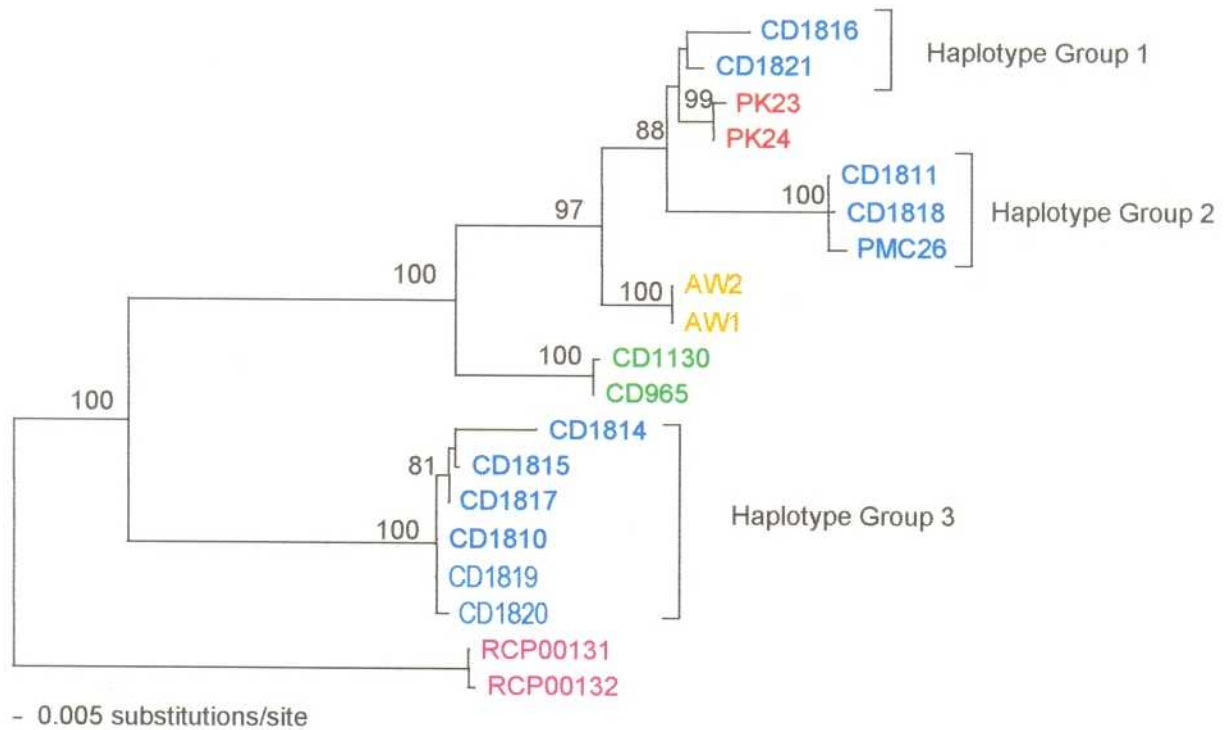
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                    1111111111111111111111111122222233333333333334444
                    78899901111225566666667777899901127802334468889 1123
                    71712401367012824578945679358218036602669703835694
CD1816   GGAAAACGTATTCCTCCTCCTTACGCCGTCTTCTCCATCCGTACTGGACT ⌉
CD1821   ......T.........T.....................T.C......TC  ⌋ HG1

CD1816   GGAAAACGTATTCCTCCTCCTTACGCCGTCTTCTCCATCCGTACTGGACT ⌉
CD1811   A..C.GTACGACTTCTGCATACCTATTACT.........T.C.....GTC │
CD1818   A..C.GTACGACTTCTGCATACCTATTACT.........T.C.....GTC │ HG2
PMC26    A..C.GTACGACTTCTGCATACCTATTACT.........T.C.....GTC ⌋

CD1816   GGAAAACGTATTCCTCCTCCTTACGCCGTCTTCTCCATCCGTACTGGACT ⌉
CD1814   AAG.G.T......T....TTC....TTAC.CCTCTTGCT.ACGTCAAG.. │
CD1815   AAG.G.T......T....TTC....TTACTCCTCTTGCT.ACGTCAAG.. │
CD1817   AAG.G.T......T....TTC....TTACTCCTCTTGCT.ACGTCAAG.. │ HG3
CD1810   AAG.G.T......T....TTC....TTACTCCTCTTGCT.ACGTCAAG.. │
CD1819   AAG.G.T......T....TTC....TTACTCCTCTTGCT.ACGTCAAG.. │
CD1820   AAG.G.T......T....TTC....TTACTCCTCTTGCT.ACGTCAAG.. ⌋


                    4444444455555555566666666666666666666666667777777888
                    3377889911122444001222344455555577778899014 6788668
                    5925671868968456357129213402345701340 1130731901293
CD1816   CCAGTCGCCGCTATCAGCCCTTTCCTTAGGCCCTTCGGCCGTACGCACAA ⌉
CD1821   ......A....................A.......A......A.....   ⌋ HG1

CD1816   CCAGTCGCCGCTATCAGCCCTTTCCTTAGGCCCTTCGGCCGTACGCACAA ⌉
CD1811   ......A..A............-........A.........          │
CD1818   ......A..A............-........A.........          │ HG2
PMC26C   ......A..A............-........A.........          ⌋

CD1816   CCAGTCGCCGCTATCAGCCCTTTCCTTAGGCCCTTCGGCCGTACGCACAA ⌉
CD1814   TTG.CTATTATCGCTGATTTCCCTTACGAATTTCCTAATGTCGTATG... │
CD1815   TTGACTATTATCGCTGATTTCCCTTACGAATTTCCTAATGTCGTATG..C │
CD1817   TTGACTATTATCGCTGATTTCCCTTACGAATTTCCTAATGTCGTATG..C │ HG3
CD1810   TTGACTATTATCGCTGATTTCCCTTACGAATTTCCTAATGTCGTATG..C │
CD1819   TTGACTATTATCGCTGATTTCCCTTACGAATTTCCTAATGTCGTATG..C │
CD1820   TTGACTATTATCGCTGATTTCCCTTACGAATTTCCTAATGTCGTATGACC ⌋


                    11111111111111111111111111111111111111111111111111
                    99999900000111111111111122222333344444444445555555
                    13589966779045577888999912345339912346689903 33469
                    97215704078591203035346846861386979826985710 47478
CD1816   TTATACGACTCACACTCATCTGTTCGGCCCCCCTATTCCCCTCCCTATG ⌉
CD1821   .C....AG...........C.............................  ⌋ HG1

CD1816   TTATACGACTCACACTCATCTGTTCGGCCCCCCTATTCCCCTCCCTATG ⌉
CD1811   .C....AGTC........C.............................   │
CD1818   .C....AGTC.T......C.............................   │ HG2
PMC26    .C....AGTC........C.............GAG-............   ⌋

CD1816   TTATACGACTCACACTCATCTGTTCGGCCCCCCTATTCCCCTCCCTATG ⌉
CD1814   .C.CCTAGTCT.TGTCTG.TCAGGTAATTTGGTAGCG.GTTCTTTAGCA │
CD1815   C.GCCTAGTCT.TGTCT..TCA..TAATT..GTAGCG.GTTCTTTCGCA │
CD1817   C.GCCTAGTCT.TGTCTG.TCA..TAATT...TAGCG.GTTCTTTCGCA │ HG3
CD1810   C.GCCTAGTCT.TGTCTG.TCA..TAATT...TAGC...TTCTTTCGCA │
CD1819   C.GCCTAGTCT.TGTCTG.TCA..TAATT...TAGC...TTCTTTCGCA │
CD1820   C.GCCTAGTCT.TGTCTG.TCA..TAATT...TAGC...TTCTTTCGCA ⌋
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Figure 1. Variable sites of aligned mitochondrial control region sequences of Forbes' Parakeet individuals showing the distinct Haplotype Groups (HG) 1-3. Control region sequence from specimen CD1816 is used as a reference for each haplogroup. Numbers above the sequences indicate the position of the variable site corresponding to positions 1-1603 of aligned sequences.

Figure 2. Maximum likelihood tree based on control region data for 19 *Cyanoramphus* individuals. C, *n. saisetti* is used as outgroup. Bootstrap values greater than 50% are indicated at nodes (500 replicates). The Hasegawa-Kishino-Yano 85 (Hasegawa *et al.* 1985) substitution model with gamma approximation of variable sites. (α= 0.239) were used. The taxon names are colour coded as follows:

Forbes' Parakeet (C. forbesi): Blue

Chatham Island Red-crowned Parakeet (C. novaezelandiae chathamensis): Red

Orange-fronted Parakeet (C. malherbi): Orange

Antipodes Island Parakeet (C. unicolor): Green

New Caledonia Red-crowned Parakeet (C. n. saisetti): Pink