APPENDIX E

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Identification of protected avian species using a single feather barb

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Journal of Forensic Sciences

Title: Identification of protected avian species using a single feather barb*

Authors: Sansook Boonseub,[1] M.Sc.; Greg Johnston,[1,2] Ph.D.; Adrian Linacre,[1]*D.Phil.

Affiliation:

[1] School of Biological Sciences, Flinders University, Adelaide, Australia

[2] School of Natural and Built Environments and Barbara Hardy Institute, University of South Australia, Adelaide, Australia

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Disclaimer: the work conducted and conclusions drawn are those of the authors and do not necessarily reflect the views of the academic institutions.

ABSTRACT

We report on the unambiguous identification of protected avian species from as little as one barb of a feather. Many avian species are protected by international agreements and national legislation, yet they are traded illegally due to their high value. Two sections of the avian mitochondrial genome were chosen to identify bird species, being a 561 bp section of ND2 gene and a 921 bp section of the ND5 gene. Two different DNA extraction methods were compared for their ability to reliably isolate sufficient to be detected in a subsequent PCR. Using a commercial kit supplied by QIAGEN a complete sequence was obtained from one barb for the ND2 gene, whereas two barbs were required to reliably sequence the 921 section of the ND5 gene. The process worked on all species tested using feathers from archival museum specimens, resulted in minimal damage to the specimen and can readily be adopted by a forensic science laboratory.

Key words: forensic science, feather, barb, calamus, avian species, ND2, ND5

Many avian species are traded illegally due to their high value. This is particularly the case for parrots (family Psittacidae) where individual specimens may attract prices of $18,000 USD (1). Numerous species of parrots, macaw and cockatoos are listed on the appendices of the Convention on the International Trade in Endangered Species of Flora and Fauna (CITES) and subject to national legislation such as the US Endangered Species Act (ESA) and the Environment Protection and Biodiversity Conservation Act in Australia (EPBC). As an example, over 40 species of parrot are listed on CITES Appendix I affording them the greatest protection and prohibiting international trade between member countries. Despite this protection, one study in Bolivia (2) showed that during a 12 month period authorities seized over 7,000 individual birds of 31 different parrot species, all of which are listed on CITES Appendix I. There was no estimate of the number of individuals traded illegally and not seized. The illegal trade of avian species in common with the trade in other protected species offers large financial benefits, with little chance of capture, and relatively minor penalties if successfully prosecuted (3).

It may be the case that only chicks are seized, in which case it may not be possible to identify the species by gross morphology, or when a single feather is the only trace indicating potential illegal trading of these protected species. Feathers are similar in structure in many regards to hair as they are composed primarily of keratin. The structure of the feather consists of a central stiff shaft from which numerous barbs extend. The proximal section of the central shaft is termed the calamus, and has been the focus of previous attempts to obtain DNA (4-10). These procedures require much destruction of the feather and are best suited to fresh material. Fresh material is atypical in forensic science as normally the samples have received some external damage or may no longer be fresh at the time of examination. One recent publication illustrated

the potential for isolating DNA from barbs (11) as there are benefits in minimal damage to the item if the feather is rare or precious.

We report on a simple method to extract from feather barbs a section of the avian mitochondrial genome suitable for species identification. A 921 bp fragment of the ND5 gene and a 561 bp fragment of the ND2 gene were amplified independently from two barbs and a single barb respectively. Barbs were taken from a range of species; samples included feathers collected over seven months prior to analysis and from a museum sample with a collection date of 1979. The amplification primers were designed to successfully amplify a product from any avian species but under the conditions used will not amplify mammalian, including human, DNA. The PCR products were sequenced and the correct avian species identified indicating that this is a suitable method for avian species identification in a forensic context when there is only one feather available and minimal destruction is preferable.

Materials and methods

Sample collection

Samples of avian species listed in Table 1 were obtained after identification of the species. We follow the taxonomic system used by Pizzey and Knight (12). An example of the size of a single barb, and feather from which it was removed, is shown in Figure 1.

DNA extraction

Two commercially available products were used in this work; the QIAGEN micro kit (QIAGEN, Doncaster, Australia)and the Promega DNA IQ kit (Promega, Sydney, Australia). As the

QIAGEN product performed better than that of the Promega, all the data in the paper relates to extracts using the QIAGEN method of DNA isolation. In both cases, individual barbs were removed from the feathers, weighed, and then placed in a 1.5 mL tube.

DNA isolation using the QIAamp® DNA micro kit

To the 1.5 mL tube, 300 µL of ATL buffer plus 20 µL of proteinase K (20 mg/mL) and 10 µL of DTT (1M) were added. The barb suspension was incubated at 56 °C for 2 hours or until the barb had dissolved completely. The procedure was then conducted as the manufacturer's recommendation with the exception that the DNA was eluted twice with 30 µL of pre-warmed (37 °C) AE to collect a final volume of 60 µL.

DNA isolation using the Promega DNA IQ kit

To the 1.5 mL tube, 259 µL of Lysis Buffer plus 10 µL of proteinase K (20 mg/mL) and 10 µL of DTT (1M) were added. The barb suspension was incubated at 56 °C for 2 hours or until the barb had dissolved completely. To this suspension, 21 µL of resin was added and the procedure was then conducted as the manufacturer's recommendation with the exception that the DNA was eluted with 30 µL of pre-warmed (37 °C) Elution Buffer.

DNA Amplification

All PCRs were conducted with a negative PCR control to monitor any contamination and a positive control of DNA from muscle tissue of domestic chicken (*Gallus gallus*).

Amplification of ND2 locus

Amplifications were performed in a volume of 25 µL containing 5 µL of Go Taq Buffer (Promega), 2 µL of 25 mM $MgCl_2$ buffer, 2 µL of 2 mM dNTPs, 1.5 µL of each primer (at 10 µM concentrations) and 2 units of Go Taq (Promega). The sequences of the primers were 5' CATACCCCGAAAATGATGGT 3' and 5' TGTGTYTGGTTKAGKCCTAT 3'. The PCRs were conducted on a MULTIGENE Labnet PCR machine using the following conditions: 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s.

Amplification of ND5 locus

Amplifications were performed in a volume of 25 µL containing 5 µL of Go Taq Buffer (Promega, Sydney, Australia), 2 µL of 25 mM $MgCl_2$ buffer, 2 µL of 2 mM dNTPs, 1.5 µL of each primer (at 10 µM concentrations) and 2 units of Go Taq (Promega). The sequences of the primers were 5' CTTGGTGCAAMTCCARGTRAAAG 3' and 5' TTGATGTCRTTTTGKGTGAGRGC 3'. The PCRs were conducted on a MULTIGENE Labnet PCR machine using the following conditions: 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s.

PCR products were separated on a 2% agarose gel and visualised using a Gel Doc™ EZ Imager (Bio-Rad, Gladesville, Australia).

PCR Purification and sequencing

The PCR product of interest was excised from the agarose gel and DNA purified using the QIAquick Gel Extraction kit (QIAGEN). The manufacturer's protocol was followed. Approximately 50 ng of purified PCR products, as determined using a NanoDrop 1000

spectrophotometer (Thermo Scientific, Scoresby, Australia), was added to 10 pmol of primer in a volume of 12 µL; this was sent for sequencing at the Australian Genome Research Facility.

DNA Sequence Comparison

The sequencing results were compared to the reference sequences on the GenBank DNA database using the Blast program (http://blast.ncbi.nlm.nih.gov/).

Results and Discussion

Amplifications were performed using 1, 2, 5, 10 and 20 barbs from a range of feathers. These data are presented in Figure 2 illustrating that a PCR product was obtained from all samples and that there was sufficient template in the extract from 1 barb to allow for subsequent full DNA sequencing.

Relatively more DNA was obtained when an increasing number of barbs were used in the extraction up to 40 barbs although when 80 barbs were used consistently less DNA was obtained (data not shown).

A PCR product of 921 bp amplified from ND5 was obtained from two barbs removed from museum specimen that was taxidermically mounted in 1979; 32 years prior to the time of analysis.

Clear and unambiguous sequence data were obtained from amplifications conducted on a single barb. These data were compared to those registered on GenBank (www.ncbi.nlm.nih.gov) or DNA sequence data obtained from voucher specimens. This comparison confirmed the species from which the feather came; in all instances the avian species could be identified. The data are shown in Figure 3a and b where a section of 569 bp from one barb taken from a short-billed

black cockatoos *Calyptorhynchus laterostris* was found to match a sequence on GenBank from the same species with a 99 % similarity. An incomplete section (404 bp) of the 921 bp section of the ND5 gene was found to have a 99 % homology to a species listed on GenBank.

Comparable quantities of DNA were extracted from varying numbers of barbs using the QIAGEN and Promega kits; however the success of amplification was routinely better for DNA extracts amplified using the QIAGEN kit indicating that the quality is better (Figure 4).

The DNA amplified by the primer sets requires a length and sequence suitable for unambiguous species identification, and in this regard the section of the ND2 gene is ideal. A larger amplification product from the ND5 gene was also obtained allowing both genes to be sequenced; as recommended recently for avian species identification (13). No contamination was noted in any reactions performed and the positive control gave the expected results. The sequence data exhibited no indication of a mixture. No indication of heteroplasmy was noted in the DNA sequence obtained. Any exogenous human DNA on the samples was not amplified by the avian species-specific primers. Specificity tests using other species including snake and human DNA was tested and no product was produced using this primer set.

The test described will be suitable for use on archived material and single feathers, where minimal damage is inflicted on the specimen. The process uses methods of DNA extraction used routinely by forensic science laboratories and would require little validation prior to use in casework. The section of the DNA amplified was chosen deliberately as the section of the ND2 gene has been found previously (14) to be suitable for avian species identification.

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Additional information and reprint requests:

Adrian Linacre, D.Phil.

Justice Chair in Forensic Science

School of Biological

Flinders University

Adelaide 5001

South Australia

adrian.linacre@flinders.edu.au

Table 1: A list of the parrots and cockatoos species including their common name

Scientific name	Common name
Calyptorhynchus latirostris	Short-billed Black Cockatoo
Nymphicus hollandicus	Cockatiel
Polytelis anthopeplus	Regent Parrot

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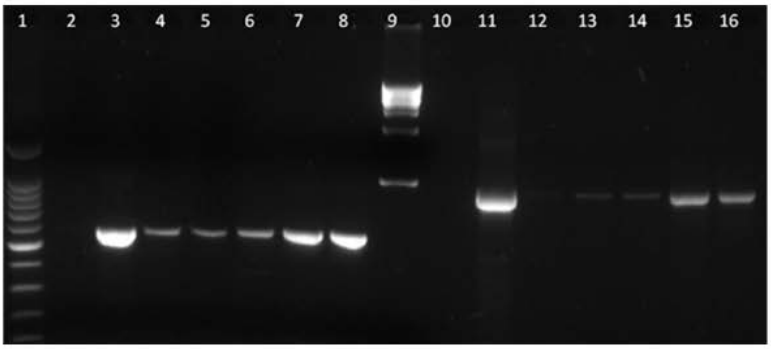

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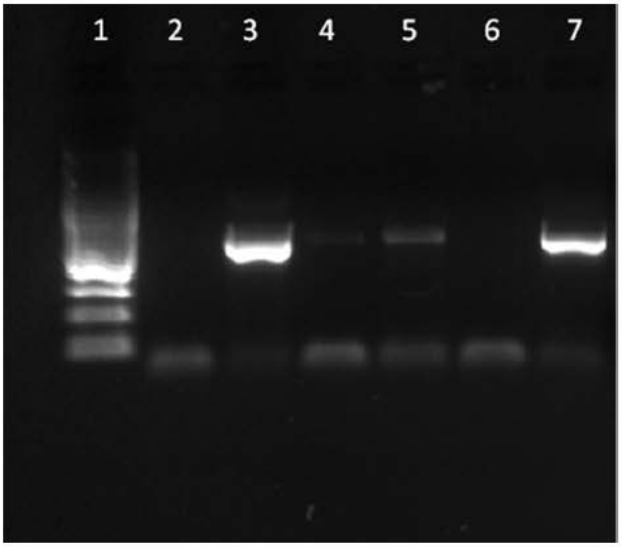

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Research article

The use of mitochondrial DNA genes to identify closely related avian species

Sansook Boonseub, Shanan S. Tobe, Adrian M.T. Linacre *

Centre for Forensic Science, WestCHEM, Department of Pure and Applied Chemistry, University of Strathclyde, 204 George Street, Glasgow G1 1XW, UK

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ABSTRACT

Species identification using mitochondrial DNA (mtDNA) loci is a standard method for mammalian species testing. Less is understood about the conservation and variability in the avian mitochondrial genome, yet many exotic bird species are threatened with extinction and are traded illegally. In this study 80 different avian species were chosen from 22 different Orders and their gene sequences for the cytochrome *b*, cytochrome oxidase I and the ND2 genes were obtained from the NCBI web site. Alignments of the sequence determined the areas of greatest variation and conservation. The alignment result of DNA sequence showed that the cytochrome *b* gene placed the most number of avian species into their appropriate Orders, ND2 was next closest and COI the poorest of the three loci. These data support the use of cytochrome *b* over the other two mitochondrial loci for avian species identification.

1. Introduction

Attention in species identification falls on mammalian species primarily [1–4] and on occasion insect species [5,6], but rarely do avian species become part of a forensic science investigation. Many avian species are protected and their trade is illegal. Parrot species and birds of prey are examples of birds that are the subject of theft and illegal trade. The mitochondrial DNA markers used most commonly in mammalian species identification are the cytochrome *b* (cyt *b*) [7,8] and cytochrome oxidase I (COI) gene sequences [7,9]. The avian mitochondrial genome is similar to the mammalian but some of the 37 genes are in a different Order, indicating that these genomes have a different recent evolutionary history [10]. The cyt *b* gene is used most commonly but more recently there has been an interest in the use of COI and ND2. This paper compares these three genes on the avian mitochondrial genome to determine which may be the better loci at differentiating between closely related avian species.

2. Materials and methods

2.1. Species identification in wide-ranged avian species

The cyt *b*, COI and ND2 DNA sequences were identified from GenBank (http://www.ncbi.nlm.nih.gov/) for 80 different avian species. The multiple sequences alignments were aligned using the ClustalW program (http://align.genome.jp/). The phylogenetic tree of each gene was created using the interactive tree of life program from http://itol.embl.de/.

3. Results

The completed mitochondrial sequences of the cyt *b* and COI and ND2 genes were obtained for 80 avian species. The species selected covered all the major avian families. The DNA sequence for each gene was aligned and a phylogenetic tree was created and analysed.

The alignment of the COI gene sequences showed that there were only 7 of the 22 Orders that grouped together, with the members of the other 15 Orders being grouped inappropriately. Using the alignment of the ND2 gene sequences there were 10 Orders of the 22 that grouped together. The cyt *b* gene placed 14 of the Orders into uninterrupted groups (see Fig. 1). The numbers around the outside of the figure indicate the 14 Orders that are grouped together.

According to previous phylogenetic studies [11], the species from the 22 different Orders in our study can be classified into eight distinct clades, as following;

(1) The passerines (perching birds), parrots, birds of prey, woodpeckers, kingfishers, trogons and owls.
(2) The shorebirds (turnstones, oystercatchers).
(3) The pelicans, storks, herons, penguins, albatross and divers.
(4) The coots, cranes, rails and cuckoos.
(5) The swifts, hummingbirds and nocturnal birds (nightjar).
(6) The tropical birds, flamingos and grebes.
(7) The megapodes, curassows, pheasants, quails, and relatives, ducks, geese, swans.
(8) The flightless bird (emu, ostrich, rhea).

* Corresponding author.
E-mail address: a.m.t.linacre@strath.ac.uk (Adrian M.T. Linacre).

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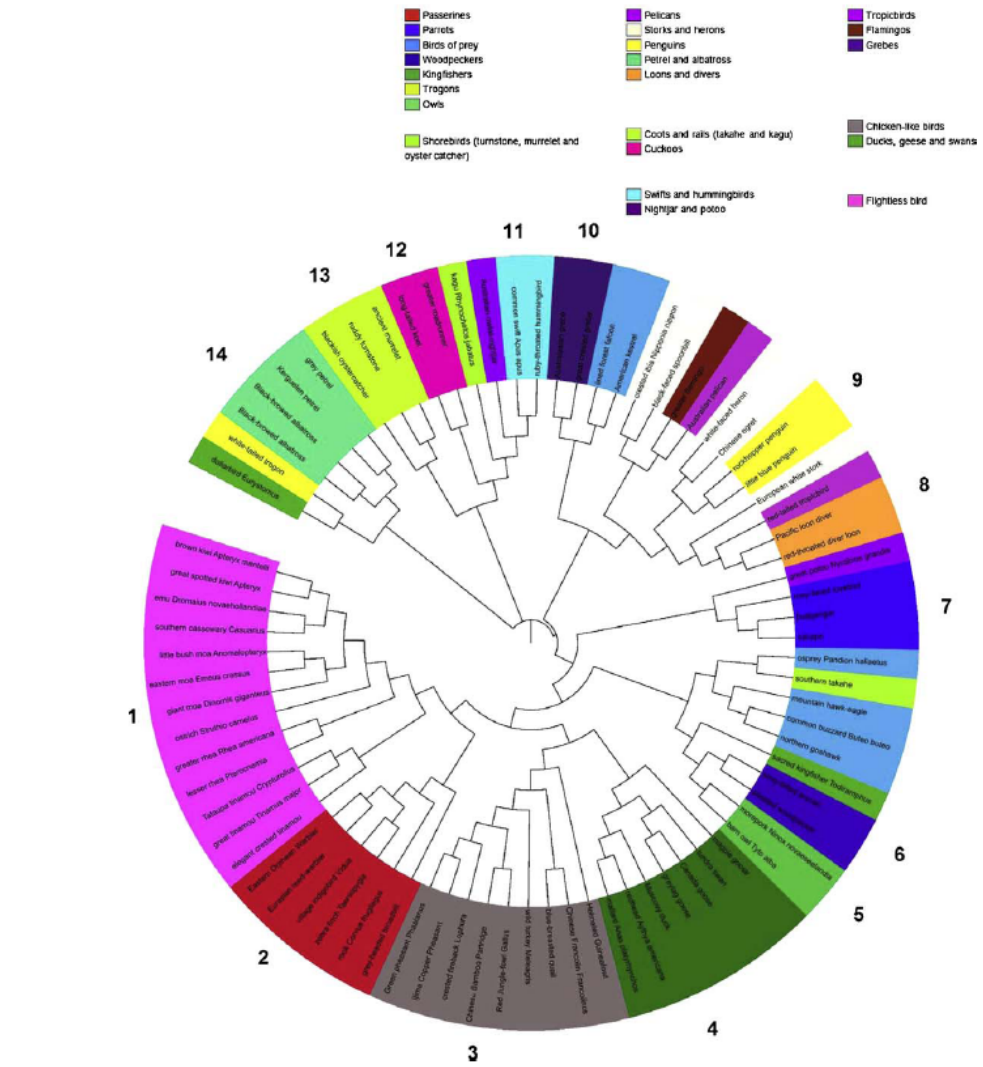

Fig. 1. The phylogenetic tree of complete cyt *b* gene sequences from 80 different avian species. The sequences were selected from GenBank which cover all the major avian families. The species are arranged based on their sequence alignments. The 22 different Orders are coded and the numbers around the outside indicate the 14 uninterrupted and correctly assigned Orders.

Using the data in Fig. 1, the cyt *b* gene groups the megapods with the swans as predicted. The same is true for linking other Orders into the same clade. Exceptions include the tropical birds that are grouped but more distantly than predicted using the cyt *b* gene. Using the 8 groups as above the cyt *b* gene produced more expected linkages and fewer anomalies compared to the ND2 or COI gene.

4. Conclusions

Our preliminary data indicate that the cyt *b* gene can separate a wide range of avian species into their respective Orders. Of the three genes tested, the ND2 produced fewer anomalies than the COI gene, which was the poorest in grouping the species. The sequences used are those of the entire gene and in forensic studies or Barcoding work only sections of the genes are used. Despite this our indication is that for avian species the cyt *b* gene remains the gene with greatest potential for accurate species identification of an unknown avian sample.

Conflict of interest

None.

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