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TECHNICAL NOTE

CRIMINALISTICS

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Identification of Protected Avian Species Using a Single Feather Barb*,†

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 because of 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 DNA 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-bp 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.

KEYWORDS: forensic science, feather, barb, calamus, avian species, ND2, ND5

Many avian species are traded illegally because of 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 in 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 U.S. Endangered Species Act and the Environment Protection and Biodiversity Conservation Act in Australia. As an example, over 40 species of parrot are listed in CITES' appendix 1 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 7000 individual birds of 31 different parrot species, all of which are listed in CITES' appendix 1. 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 7 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 polymerase chain reaction (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 the 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 Fig. 1.

DNA Extraction

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

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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

the QIAGEN product performed better than that of Promega, all the data in the study relate 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 tissue lysis buffer (ATL) plus 20 μL of proteinase K (20 mg/mL) and 10 μL of DTT (1 M) were added. The barb suspension was incubated at 56°C for 2 h or until the barb had dissolved completely. The procedure was then conducted according to the manufacturer's recommendation with the exception that the DNA was eluted twice with 30 μL of prewarmed (37°C) elution buffer (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 ATL plus 10 μL of proteinase K (20 mg/mL) and 10 μL of DTT (1 M) were added. The barb suspension was incubated at 56°C for 2 h or until the barb had dissolved completely. To this suspension, 21 μL of resin was added, and the procedure was then conducted according to the manufacturer's recommendation with the exception that the DNA was eluted with 30 μL of prewarmed (37°C) AE.

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′ TGTGTYT GGTTKAGKCCTAT 3′. The PCRs were conducted on a MULTIGENE Labnet PCR machine using the following conditions: 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min 30 sec.

Amplification of ND5 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′ 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 sec, 55°C for 30 sec, and 72°C for 1 min 30 sec.

PCR products were separated on a 2% agarose gel and visualized using a Gel Doc™ EZ Imager (Bio-Rad, Gladesville, NSW, 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, Vic., 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 Fig. 2,

FIG. 1—*Showing an example of a single barb and the feather from which it was taken. The larger feather and barb (bottom) is from the Short-billed Black Cockatoo* (Calyptorhynchus latirostris)*, the Regent Parrot* (Polytelis anthopeplus) *is shown top right, and Cockatiel* (Nymphicus hollandicus) *is shown top left.*

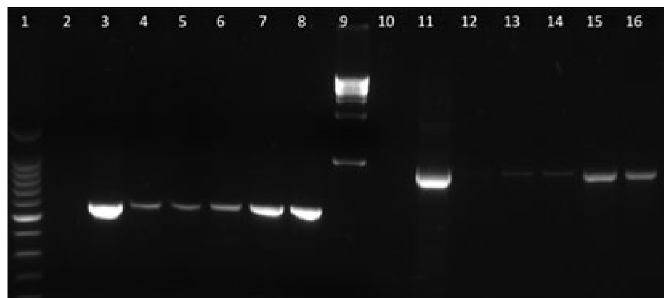

FIG. 2—*Showing amplification of sections of the avian mitochondrial ND2 and ND5 genes from the Short-billed Black Cockatoo (*Calyptorhynchus latirostris*). Lanes 1–8 are amplification from the ND2 gene producing a product of 561 bp and are, left to right, 100-bp marker, negative control, positive control from chicken muscle, 1 barb, 2 barbs, 5 barbs, 10 barbs and 20 barbs. Lanes 9–16 are amplifications from the ND5 gene producing a product of 921 bp and are left to right, 1 kb marker, negative control, positive control from chicken muscle, 1 barb, 2 barbs, 5 barbs, 10 barbs and 20 barbs.*

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(a)
Query  2     CCTCTTCT-ATGAG-CCACTCAC-AAACTAACCCTAACACTCAGCCTAACCCTAGGGACT  58
             ||||| || ||||| || ||||| ||||||||||||||||||||||||||||||||||||
Sbjct  3991  CCTCTACTAATGAGCCCCCTCACAAAACTAACCCTAACACTCAGCCTAACCCTAGGGACT  4050

Query  59    ACAACCACAATCACAAGCAGCCACTGAGTAATAGCCTGAATCGGGCTAGAAATCAATACC  118
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4051  ACAACCACAATCACAAGCAGCCACTGAGTAATAGCCTGAATCGGGCTAGAAATCAATACC  4110

Query  119   CTAGCTATAATCCCCCTAATCTCAAAATCTCACCACCCCCGAGCCACCGAAGCAGCAACC  178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4111  CTAGCTATAATCCCCCTAATCTCAAAATCTCACCACCCCCGAGCCACCGAAGCAGCAACC  4170

Query  179   AAGTACTTCCTAGTACAAGCAACTGCTTCAACACTGATACTCTTCTCGAGCATAACCAAT  238
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4171  AAGTACTTCCTAGTACAAGCAACTGCTTCAACACTGATACTCTTCTCGAGCATAACCAAT  4230

Query  239   GCATGGTCCTCCGGACAATGAGACATCACCCAACTCACCAACCCCCCATCATGCATCCTA  298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4231  GCATGGTCCTCCGGACAATGAGACATCACCCAACTCACCAACCCCCCATCATGCATCCTA  4290

Query  299   CTAACTACTGCAATTGCCATTAAACTAGGACTAACCCCATTTCACTTCTGATTCCCAGAA  358
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4291  CTAACTACTGCAATTGCCATTAAACTAGGACTAACCCCATTTCACTTCTGATTCCCAGAA  4350

Query  359   GTCCTACAAGGCTCATCCCTCATTACAGCCCTACTACTCTCAACAGCAATAAAACTCCCA  418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4351  GTCCTACAAGGCTCATCCCTCATTACAGCCCTACTACTCTCAACAGCAATAAAACTCCCA  4410

Query  419   CCAATTACCATCCTCCTCCTCACATCACACTCACTAAACCCCACACTACTTACCATCCTA  478
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4411  CCAATTACCATCCTCCTCCTCACATCACACTCACTAAACCCCACACTACTTACCATCCTA  4470

Query  479   GCTGTCATATCCATTGCTCTAGGTGGTTGAATAGGCCTCAACCAAACACAA  529
             ||||||||||||||||||||||||||||||||||| || ||||||||||||
Sbjct  4471  GCTGTCATATCCATTGCTCTAGGTGGTTGAATAGGACTTAACCAAACACAA  4521

(b)
Query  33    ACACTAACCATCGTCCTAACACCTACATTCCTCCCTCTCCT-CTCAAAAAACCTCCAAAA  91
             |||||||| ||| |||| |||||||| |||||||||||||| || ||||||| |||||||
Sbjct  11864 ACACTAACAATCATCCTCACACCTACCTTCCTCCCTCTCCTACT-AAAAAACTTCCAAAA  11922

Query  92    CTC-CCCTAAAACCATCACCCTCACTATTAAATCCGCATTCCTAACCAGTC-TAGTACCA  149
             ||| ||| |||||||||||||| ||||| ||| | || |||||||| || | || |||||
Sbjct  11923 CTCTCCC-AAAACCATCACCCTTACTATCAAAACTGCCTTCCTAACTAG-CTTAATACCA  11980

Query  150   ACAACAATCTTTATACAATCAGGACTAGACAACATCACCTCATACTGAGAATGAAAGTTT  209
             |||||| |||| ||||| |||||| ||||||| ||||| ||| ||||||||||||| |||
Sbjct  11981 ACAACACTCTTCATACACTCAGGATTAGACAATATCACTTCACACTGAGAATGAAAATTT  12040

Query  210   ATCATAAACTTCAAAA-TCCCTATCAGCCTTAAAATAGACCAATACTCCATACTATTTCT  268
             |||||||| || |||| ||| | ||||| |||||||||||||||||||||||||||| ||
Sbjct  12041 ATCATAAATTTTAAAATTCCAT-TCAGCTTTAAAATAGACCAATACTCCATACTATT-CT  12098

Query  269   -CCCTACCGCCCTATTCGTAACATGATCTATCCTACAATTTGCAATATCATACATAAAAT  327
              ||||| ||| ||||| || ||||||||||| |||||||||||||  |||||||||   |
Sbjct  12099 TCCCTATCGCACTATTTGTGACATGATCTATTCTACAATTTGCAACCTCATACATAGCCT  12158

Query  328   CCGACCCACACATTACAAAATTCTTCTCCTACCTAACAACCTTTCT-AATCGCAATATTA  386
             | ||||||||||| ||||||||||||||||| ||||||||||| || |||||| ||| ||
Sbjct  12159 CAGACCCACACATCACAAAATTCTTCTCCTATCTAACAACCTT-CTTAATCGCTATACTA  12217

Query  387   ACACTAACCCTCGCCAACAACATCTTCCTCCTCTTCATTGGTTGAGAAGGAGTAGGCATT  446
             ||| ||||||| || ||||| || ||||||||||||||||| |||||||||||||| ||
Sbjct  12218 ACATTAACCCTTGCTAACAATATATTCCTCCTCTTCATTGGCTGAGAAGGAGTAGGTATC  12277

Query  447   ATATCCTTCCTACTAATCAGCTGATGACA  475
             ||||||||| || ||||||||||||||||
Sbjct  12278 ATATCCTTCTTATTAATCAGCTGATGACA  12306
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FIG. 3—*(a) Showing an example of the comparison sequence data from one barb after amplification of ND2. The complete 561-bp fragment was sequenced and found to match that of the Short-billed White-tailed Black Cockatoo* (Calyptorhynchus latirostris*) (accession number JF414243) with a similarity of 99%. (b) Showing an example of the comparison sequence data from two barbs after amplification of DNA of a 921-bp fragment of ND5. A partial sequence of 475 bp was found to have a similarity of 86% to* Aprosmictus erythropterus *(accession number AY309456).*

illustrating that a PCR product was obtained from all samples and that there was sufficient template in the extract from one 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 (http://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 Fig. 3*a*,*b* where a section of 569 bp from one barb taken from a Short-billed Black Cockatoo (*Calyptorhynchus latirostris*) was found to match a sequence on GenBank from the same species with a 99% similarity. An incomplete section (475 bp) of the 921-bp section of the ND5 gene was found to have an 86% homology to a species listed in 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 (Fig. 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

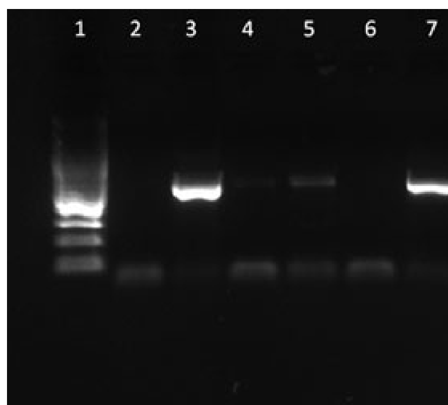

FIG. 4—*Showing the comparison of PCR products after amplifying DNA extracted from QIAGEN and Promega products using the ND2 primer sets. Lane 1 is the 100-bp marker; lane 2 is a negative PCR control; lane 3 is a positive control; lane 4 is from 10 barbs of the Cockatiel* (Nymphicus hollandicus) *sample using the DNA-IQ; lane 5 is 10 barbs of the Cockatiel sample using the QIAGEN micro kit; lane 6 is 10 barbs from the Regent Parrot* (Polytelis anthopeplus) *using the DNA-IQ; and lane 7 is 10 barbs from the Regent Parrot using QIAGEN micro kit. The example shows a greater amount of PCR product at the expected size when DNA was extracted using the QIAGEN kit compared to the Promega DNA-IQ kit; these same data were observed when using fewer barbs and when using the ND5 primer sets.*

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 were 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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