

CHAPTER 4 IDENTIFICATION OF PROTECTED AVIAN SPECIES USING A SINGLE BARB

As this chapter is prepared for publication in peer-reviewed journals, two people other than myself have contributed to the work. These are Adrian Linacre at Flinders University who supervised the work and co-authored the paper and Greg Johnston who helped collect samples and co-authored the paper.

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, a 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 is 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 as shown in Figure 4.1, and has been the focus of previous attempts to obtain DNA [4-11]. 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. Additionally there are benefits in minimal damage to the item if the feather is rare or precious.



Figure 4.1: showing the calamus section of the bird feather.

This chapter reports 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. The optimum temperature for amplifying the ND2 and the ND5 segments are shown in Figure 4.2a and 4.2b. The optimum temperature for both primer pairs are at 52 °C.

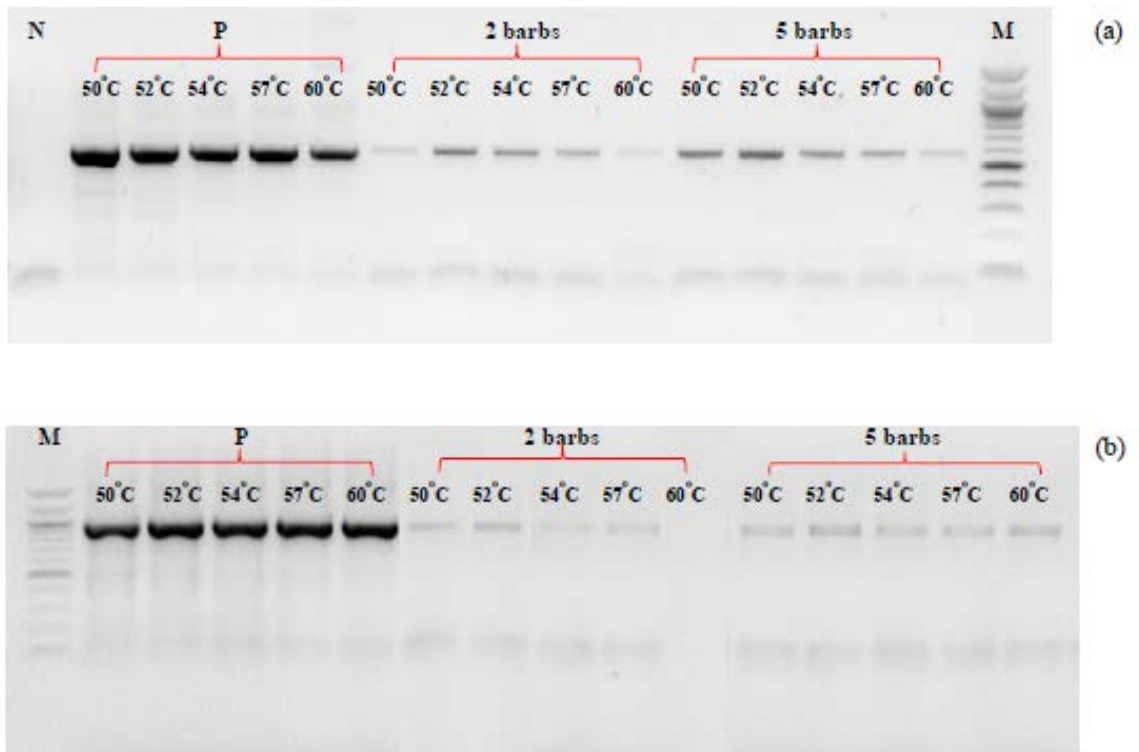


Figure 4.2: showing amplification of sections of the avian mitochondrial ND2 and ND5 genes from 2 barbs and 5 barbs of the Short-billed Black Cockatoo (*Calyptorhynchus latirostris*) under various annealing temperature of 50°C, 52°C, 54°C, 57°C and 60°C. The amplification from the ND2 gene producing a product of 561 bp is in Figure 4.2(a) and M is a 100 bp marker, N is a negative control, P is a positive control from chicken muscle. The amplification from the ND5 gene producing a product of 921 bp is in Figure 4.2(b) and M is a 1 kb marker, N is a negative control, P is a positive control from chicken muscle.

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. An example of the size of a single barb, and feather from which it was removed, is shown in Figure 4.3.



Figure 4.3: 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.

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. Amplifications were performed using 1, 2, 5, 10 and 20 barbs from a range of feathers. These data are presented in Figure 4.4. A PCR product can be obtained from all samples with sufficient template in the extract from 1 barb for full DNA sequencing.

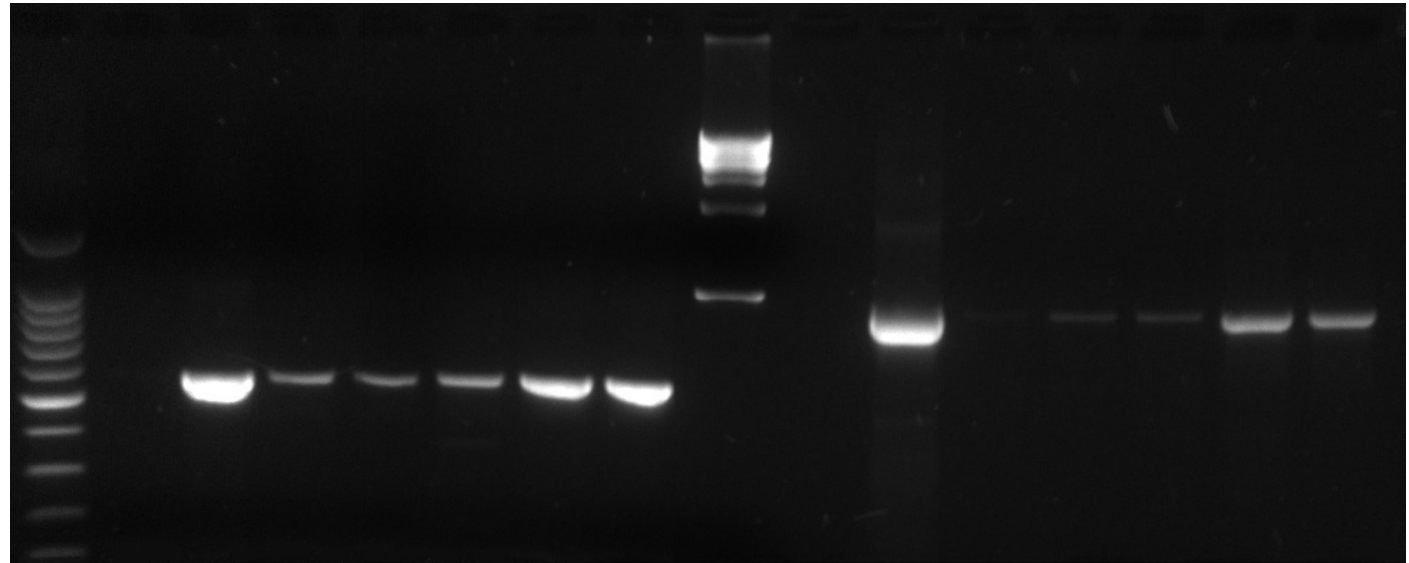


Figure 4.4: showing amplification of sections of the avian mitochondrial ND2 and ND5 genes from the Short-billed Black Cockatoo (*Calyptorhynchus latirostris*). Lanes 1-8 are amplification from the ND2 gene producing a product of 561 bp and are, left to right, 100 bp marker, negative control, positive 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.

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 as shown in Figure 4.5.

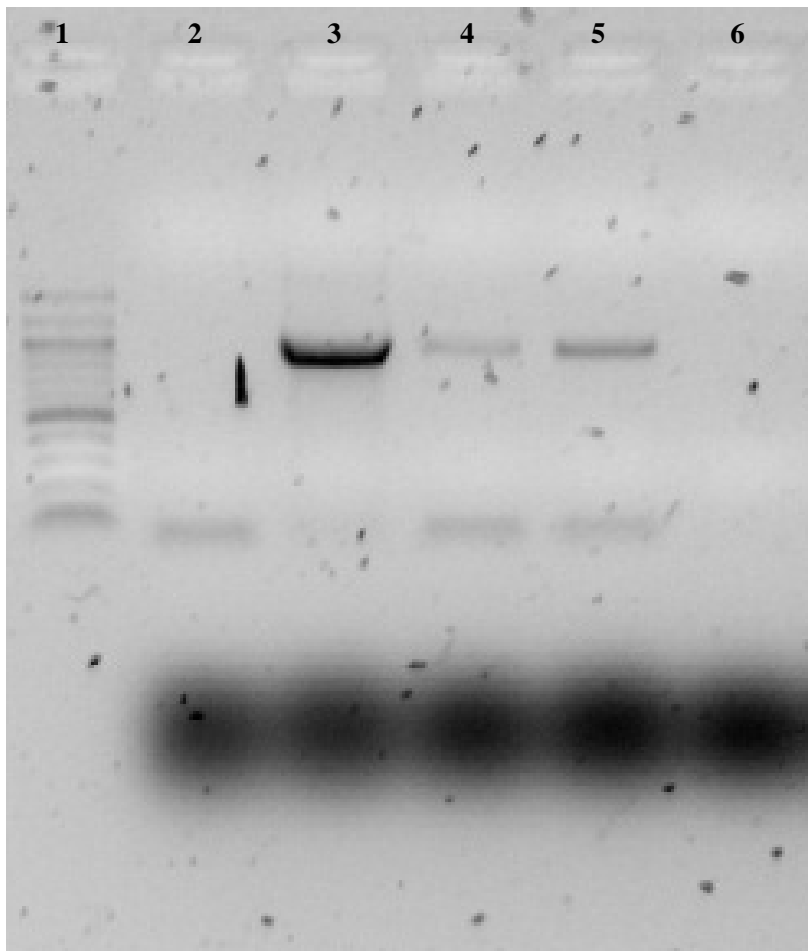


Figure 4.5: showing amplification of sections of the avian mitochondrial ND2 and ND5 genes from the Superb Parrot (*Polytelis swainsonii*). Lanes 1-6 are amplification from the ND5 gene producing a product of 921 bp and are, left to right, 1 kb marker, negative control, positive control from chicken muscle, 10 barb, 40 barbs and 80 barbs.

A PCR product of 921 bp amplified from ND5 was obtained from two barbs removed from museum specimen that was taxidermically mounted in 1979; this being 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. Examples of the data are shown in Figure 4.6a and 4.6b 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.

Query	2	CCTCTTCT-ATGAG-CCACTCAC-AAACTAACCCCTAACACTCAGCCTAACCCCTAGGGACT	58
Sbjct	3991	CCTCTACTAATGAGCCCCCTCACAAAATAACCCTAACACTCAGCCTAACCCCTAGGGACT	4050
Query	59	ACAACCACAATCACAAGCAGCCACTGAGTAATAGCCTGAATCGGGCTAGAAATCAATACC	118
Sbjct	4051	ACAACCACAATCACAAGCAGCCACTGAGTAATAGCCTGAATCGGGCTAGAAATCAATACC	4110
Query	119	CTAGCTATAATCCCCCTAATCTCAAATCTCACACCCCGAGCCACCGAAGCAGCAACC	178
Sbjct	4111	CTAGCTATAATCCCCCTAATCTCAAATCTCACACCCCGAGCCACCGAAGCAGCAACC	4170
Query	179	AAGTACTTCCTAGTACAAGCAACTGCTTCAACACTGATACTCTTCTCGAGCATAACCAAT	238
Sbjct	4171	AAGTACTTCCTAGTACAAGCAACTGCTTCAACACTGATACTCTTCTCGAGCATAACCAAT	4230
Query	239	GCATGGTCCTCCGGACAATGAGACATCACCCAACCTCACCAACCCCCCATCATGCATCCTA	298
Sbjct	4231	GCATGGTCCTCCGGACAATGAGACATCACCCAACCTCACCAACCCCCCATCATGCATCCTA	4290
Query	299	CTAACTACTGCAATTGCCATTAAACTAGGACTAAGCCATTTCACTTCTGATTCCCAGAA	358
Sbjct	4291	CTAACTACTGCAATTGCCATTAAACTAGGACTAAGCCATTTCACTTCTGATTCCCAGAA	4350
Query	359	GTCCTACAAGGCTCATCCCTCATTACAGCCCTACTACTCTCAACAGCAATAAACTCCCA	418
Sbjct	4351	GTCCTACAAGGCTCATCCCTCATTACAGCCCTACTACTCTCAACAGCAATAAACTCCCA	4410
Query	419	CCAATTACCATCCTCCTCCTCACATCACACTCACTAAACCCCACACTACTTACCATCCTA	478
Sbjct	4411	CCAATTACCATCCTCCTCCTCCTCACATCACACTCACTAAACCCCACACTACTTACCATCCTA	4470
Query	479	GCTGTCATATCCATTGCTCTAGGTGGTTGAATAGGCCTCAACCAAACACAA 529	
Sbjct	4471	GCTGTCATATCCATTGCTCTAGGTGGTTGAATAGGACTTAACCAAACACAA 4521	

Figure 4.6a: 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 Black Cockatoo (*Calyptorhynchus latirostris*) (accession number JF414243) with a similarity of 99%.

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Query 25      CTACCATACTGCTTACACTAACAATCATTTTAAAGGCCACATTCCTCCCCCTATTACTAA 84
          |||
Sbjct 11883   CTACCATACTGCTTACACTAACAATCATTTTAAAGGCCACATTCCTCCCCCTATTACTAA 11942

Query 85      AAAACTTCCAAAACCTCCCCAAAACCATCACTACCACCATCAAAAAGCCTTCTTAACCA 144
          |||
Sbjct 11943   AAAACTTCCAAAACCTCCCCAAAACCATCACTACCACCATCAAAAAGCCTTCTTAACCA 12002

Query 145     GCTTAGTACCAACAACACTCTTTATACACTCAGGACTAGAAAAGTATCACCTCATACTGAG 204
          |||
Sbjct 12003   GCTTAGTACCAACAACACTCTTTATACACTCAGGACTAGAAAAGTATCACCTCATACTGAG 12062

Query 205     AATGAAAATTCACCACAAACTTTAAAATTCCACTTAGCTTTAAGATGGATCAGTACTCCA 264
          |||
Sbjct 12063   AATGAAAATTCACCACAAACTTTAAAATTCCACTTAGCTTTAAGATAGATCAGTACTCCA 12122

Query 265     TACTATCTTTTCCCATCGCACTGTTTCGTAACCTGATCCATTTACAATTTGCAATATCAT 324
          |||
Sbjct 12123   TACTATCTTTTCCCATCGCACTGTTTCGTAACCTGATCCATTTACAATTTGCAATATCAT 12182

Query 325     ACATAGCATCGGACCCACATCACAAAATTTCTCTTACCTAACAACATTGCTAACTG 384
          |||
Sbjct 12183   ACATAGCATCAGACCCACATCACAAAATTTCTCTTACCTAACAACATTGCTAACTG 12242

Query 385     CTATACTAACACTAACCTC 404
          |||
Sbjct 12243   CTATACTAACACTAACCTC 12262

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Figure 4.6b: 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 404 bp was found to match the Short-billed Black Cockatoo (*Calyptorhynchus latirostris*) (accession number JF414243) with a similarity of 99%.

The success of sequence data comparison is associated with the reference sequences on the database. The main problem is the ND2 (561 bp) and ND5 (921 bp) segments that have been submitted on GenBank are not available for many avian species. In such instances Blast program will match the query sequence to the closest species instead, as shown in Figure 4.7. The comparison of an incomplete section (475 bp) of the 921 bp section from a Red-Winged Parrot (*Aprosmictus erythropterus*) was found to match a sequence on GenBank from a kakapo (*Strigops habroptilus*), which is in the same member of family (Psittacidae family), with an 86% similarity. No sequences to this was of a Red-Wing Parrot were available at the time of comparison.

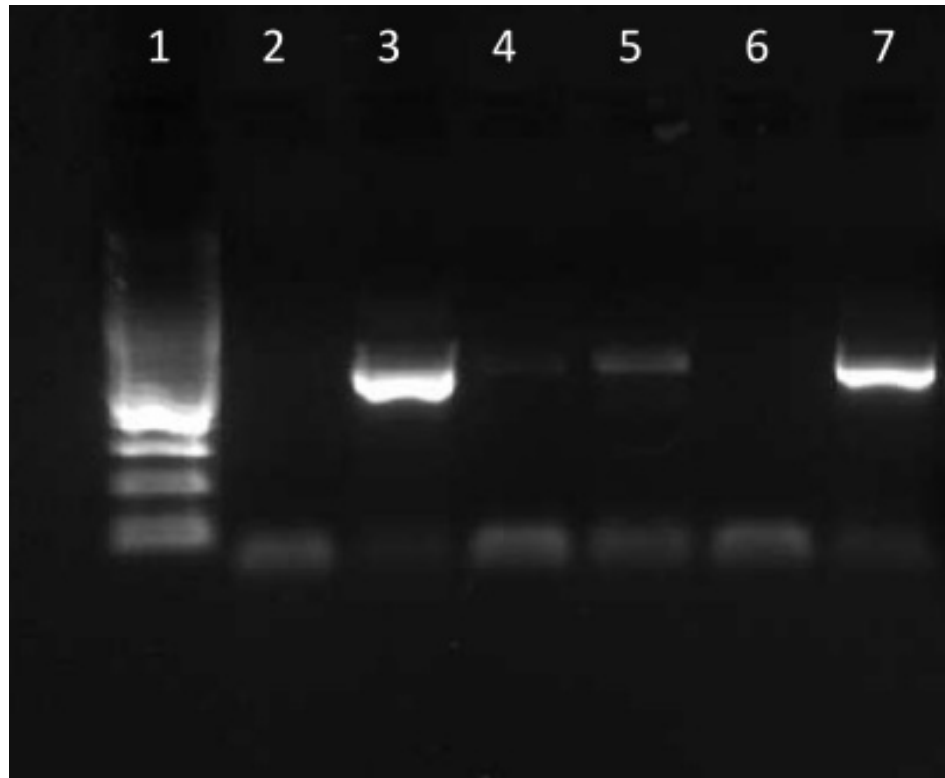


Figure 4.8: showing the comparison of PCR products after amplifying DNA extracted from QIAGEN and Promega products using the ND2 primer sets. Lane 1 is the 100 bp marker; lane 2 is a negative PCR control; lane 3 is a positive control; lane 4 is from 10 barbs of the Cockatiel (*Nymphicus hallandicus*) 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.

In summary, 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 matches this criterion. A larger amplification product from the ND₅ gene was also obtained allowing both genes to be sequenced; as recommended recently for avian species identification [12]. No contamination was noted in any reactions performed and the positive control gave the expected results.

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 [13] to be suitable for avian species identification.

Chapter 4 References

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