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BARCODING

Conserved primers for DNA barcoding historical and modern samples from New Zealand and Antarctic Birds

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Abstract

Our ability to DNA barcode the birds of the world is based on the effective amplification and sequencing of a 648 base pair (bp) region of the mitochondrial cytochrome *c* oxidase (COI or *cox*1) gene. For many geographic regions the large numbers of vouchered specimens necessary for the construction of a DNA barcoding database have already been collected and are available in museums and other institutions. However, many of these specimens are old (>20 years) and are stored as either fixed study skins or dried skeletons. DNA extracted from such historical samples is typically degraded and, generally, only short DNA fragments can be recovered from such specimens making the recovery of the barcoding region as a single fragment difficult. We report two sets of conserved primers that allow the amplification of the entire DNA barcoding region in either three or five overlapping fragments. These primer sets allow the recovery of DNA barcodes from valuable historical specimens that in many cases are unique in that they are unable or unlikely to be collected again. We also report three new primers that in combination allow the effective amplification from modern samples of the entire DNA barcoding region as a single DNA fragment for 17 orders of Southern Hemisphere birds.

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Introduction

At the heart of DNA barcoding is the proposition that individuals can be rapidly and easily assigned to the species level using standardised DNA sequences from the mitochondrial genome (Hebert *et al.* 2003a; Hebert *et al.* 2003b). A considerable body of research has been carried out that provides support for this proposition and there is currently intense international interest in this area of research. Furthermore, it has been suggested that the DNA barcoding approach, if successful, will complement modern taxonomy while at the same time revolutionising the way we routinely identify species in the future (Hajibabaei *et al.* 2007).

The World's avian fauna has been identified as a good candidate to test the general applicability of DNA barcoding because birds have been extensively studied using a range of approaches including morphology, genetics and behaviour. This has resulted in a stable and mature taxonomy (Baker *et al.* 2009; Gill 2007). However, to date, the majority of DNA barcoding studies on birds have been conducted using modern samples from Northern Hemisphere species (Hebert *et al.* 2004; Kerr *et al.* 2007; Yoo *et al.* 2006). It remains to be seen whether DNA barcoding will accurately identify species from non-continental regions where biogeographic patterns, mutation and speciation rates may have been quite different (Moritz & Cicero 2004).

New Zealand and the Antarctic have a distinctive avian fauna of moderate size (comprising of approximately 290 native or indigenous species) with a high level of endemism (Heather & Robertson 2005). In order to establish a DNA barcode database of these birds it is necessary to have access to a large number of vouchered museum specimens and to effectively amplify and sequence the DNA barcoding region from samples obtained from these specimens.

A 648bp region at the 5' end of the mitochondrial gene cytochrome *c* oxidase subunit 1 (COI or *cox*1) has been identified as the DNA barcoding region for most animal species, including birds (Hebert *et al.* 2003a; Hebert *et al.* 2004). Two types of samples are available from vouchered museum specimens, fresh or modern tissue (blood, feather or muscle that has been

preserved at low temperatures or in ethanol) and historical specimens (usually study skins or skeletons). Although many museums now collect and preserve samples from vouchered specimens for biochemical and DNA analysis (Arctander & Fjeldsa 1994; Seutin & Boag 1991), this is a relatively recent practice. As a result, there are only a relatively small number of modern tissue samples available from vouchered specimens for New Zealand and Antarctic birds and this is likely to be the case for many regions worldwide. Furthermore, the collection of new or additional vouchered specimens is in many cases difficult, expensive and time consuming. The vast majority of historical specimens are older than 20 years in age and have not been preserved with DNA analyses in mind. Samples from these specimens typically produce highly degraded DNA (Lindahl 1993). The size of the DNA fragments recovered from historical samples depends on the preservation method, sample age and a number of environmental factors (Hofreiter *et al.* 2001). The degraded nature of the recovered DNA from historical samples makes it difficult to amplify a fragment larger than 300-400bp in length as well as resulting in low rates of amplification (Zimmermann *et al.* 2008).

There are a number of approaches used to tackle the problem of generating barcodes from historical museum specimens. Short or minimalist barcodes have been recovered by amplifying only a selected 100-200bp fragment of the originally proposed ~650bp COI region. In a number of insect genera mini-barcodes have proved to be successful in identifying almost all species (Hajibabaei *et al.* 2006). However, mini-barcodes show limited success compared with the full-length barcode and only work within confined taxonomic groups (Hajibabaei *et al.* 2006). Although potentially useful for the identification of individual samples, this approach does not resolve the issue of having to construct an initial full-length database from which to make these identifications. Furthermore, whether it can be applied to avian species is yet to be determined.

Another approach is to use a range of DNA repair enzymes. These enzymes can increase the amplification success rate by repairing some of the damage that is induced in DNA as a result of chemical treatment or aging. The most common types of DNA damage to historical and ancient DNA are oxidative and hydrolytic damage (Hoss *et al.* 1996). Deamination, a type of hydrolytic damage common to cytosine residues, results in the replacement of cytosine with uracil, ending in C to T and G to A transitions during PCR (Evans 2007). Treatment with DNA repair enzymes can result in the reversal of most types of DNA damage leading to higher amplification success rates (Evans 2007). However, this approach has a number of

shortcomings specifically its inability to repair fragmented DNA and its high cost when processing large numbers of specimens.

The ability to recover DNA sequences from historical samples is in part dependent on the size of the PCR product targeted, generally the smaller the amplicon size the higher the rate of amplification success. Therefore, for any section of DNA, complete sequences can be recovered from historical samples by targeting a number of smaller overlapping regions. Once obtained, these overlapping fragments can be assembled and this approach has been used very successfully in the study of ancient DNA (Millar *et al.* 2008). Hence, the ability to DNA barcode the birds of the World will be facilitated by the availability of a set of conserved primers that will allow the amplification of the COI barcoding region from historical samples.

We report the development of two sets of PCR primers that reliably amplify from historical samples the COI barcoding region in three or five short segments. In addition, one of these primers, in combination with two others, also allows the effective amplification of the entire COI barcoding region as a single fragment from modern samples from 17 orders of Southern Hemisphere birds tested.

Materials and Methods

Primer Design

Primers for the amplification of historical and modern tissue were designed based on an alignment (using the Clustal X algorithm) of sequences obtained from New Zealand and Antarctic avian species in addition to a number of COI sequences available on GenBank (accession numbers available in supplementary data Table S1). Highly conserved regions were targeted as areas of interest for the synthesis of potential primers. Primer integrity was checked using Amplify 3X, available from http://engels.genetics.wisc.edu/amplify/ and NetPrimer available on the PREMIER biosoft International website www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html

Two sets of internal primers (Table 1) were designed to span the 648bp COI region for historical samples considered to have low quality DNA. The first primer set contained three primer pairs resulting in three overlapping fragments (average length ~310bp; Fig. 1). The second primer set contained five primer pairs resulting in five shorter overlapping fragments (average length ~ 190bp; Fig 1) to span the same 648bp region. Each internal primer contained a randomly generated 18bp tag attached to its 5' end in order to improve the sequencing of the resulting PCR products. Forward internal primers all contained an identical Ftag sequence and similarly all reverse internal primers contained an identical Rtag sequence. Internal primer pairs were tested on a range of modern samples represented from 15 avian orders prior to being used to amplify DNA from historical samples.

Three primers, one forward and two reverse were designed to amplify the entire 648bp COI region as a single fragment to be used for modern specimens. The forward primer, AWCF1 was also used as part of the primer set for historical samples. Primer pairs intended for modern specimens did not contain the 18bp tags. All primer sequences and their expected fragment lengths are shown in Table 1.

DNA Extraction from historical and modern samples

Historical specimens in the form of dried skins used for this study were obtained from Canterbury Museum (Christchurch, New Zealand). Until 1970 all specimens were preserved with arsenical soap. After this date the preservative used was changed to borax. Both methods are commonly used in museums worldwide. Preparation did not involve any special preservation on the legs and toe pads however. These were allowed to dry out naturally (P Scofield 2009, pers. comm. 6 April,). All samples were collected from the foot and toe region of the specimens.

Between 5 and 64mg of tissue was used per extraction and all tissue was macerated with a sterile scalpel blade prior to extraction. DNA isolation from dried skins was performed using proteinase K/DTT/SDS digestion followed by a DNeasy tissue extraction kit for the post-lysis steps according to the manufacturer's instructions (Qiagen). Negative controls were included for each set of extractions. No more than five samples were extracted per set. All pre-PCR steps were carried out in a separate laboratory solely dedicated to ancient DNA work. DNA from modern samples (fresh tissue) was extracted in a different laboratory using a standard

proteinase K digestion and a modified version of the phenol/chloroform method (Lawrence *et al.* 2008; Sambrook *et al.* 1989).

PCR amplification and Sequencing

PCRs were run in 25µl volume reactions and included 1X PCR buffer (Invitrogen), 2.5mM $MgCl_2$, 1mg/ml BSA, 0.5µM forward and reverse primers, 4% DMSO (only for 5'-tagged primers), 0.2mM dNTPs, 1U of Platinum *Taq* DNA polymerase (Invitrogen) and 3-4µl of DNA template from the final elution for historical samples and 25-50ng of DNA for modern samples. The thermal cycling conditions for historical samples was as follows: 2 min at 94°C followed by 10 cycles of 20s at 94°C, 20s at 55°C and 20s at 72°C followed in turn by 30 cycles of 20s at 94°C, 20s at 50°C and 20s at 72°C and a final 4 min at 72°C. In the case of modern samples the thermal cycling conditions were altered and consisted of 2 min at 94°C followed by 35 cycles of 30s at 94°C, 30s at 57.5°C and 30s at 72°C and a final 4 min at 72°C. PCR products were visualized on a 2% agarose gel. The remaining volume of PCR products were purified using the Agencourt AMPure PCR Purification system (Beckman Coulter). All purified PCR products were then cycle sequenced using Big Dye 3.1 chemistry and subsequently analysed on an ABI Prism 3130xl genetic analyzer. Sequencing was carried out bidirectionally to produce an overlap of at least 648bp of the COI region for all modern samples. In the case of the historical samples, three or five overlapping fragments were amplified which covered the same COI region. In each case, these fragments were bidirectionally sequenced from independent PCR reactions. Once the fragments were sequenced, the complete DNA barcode for each historical sample was assembled by concatenating sequences using the software programmes Sequencher 4.6 (Gene Codes Corporation) and Vector NTI (Invitrogen). Only those sequences which reached PHRED scores of 20 or above for at least 90% of individual bases were deemed acceptable.

Results

We designed and tested two sets of 5'-tagged PCR primers that reliably amplified short DNA fragments from historical samples for the barcoding region. The first set of primers containing three primer pairs generated three fragments approximately 310bp in size whereas the second set containing five primer pairs generated shorter fragments of approximately

190bp in size. Both primer sets were first tested on a range of modern samples with representative species from 15 different orders (Table 2) prior to be being used on historical specimens. Of a total of 29 species, both primer sets worked on all species tested.

Twenty seven historical samples were utilized in this study belonging to nine different orders (See supporting information Table S2 for species list). The age of the historical specimens tested ranged from 16 to 115 years. PCR products were amplified at similar success rates from both the three and five primer pair sets for the same given samples tested suggesting that the three primer pair set is the best option (Table 2). The AWCF1/AWCR6 primer pair which amplifies the entire 648bp COI fragment was used as a control for historical specimens with no products being amplified.

Of the 27 historical samples tested, 16 samples successfully amplified the regions of interest resulting in an amplification success rate of 59%. Further analysis found PCR success to be strongly correlated with specimen age. When separated into age groups we found samples which were 80 years or less in age had a higher average amplification success rate of 77% (n=13) than their older counterparts of greater than 80 years of age which had an average amplification success rate of 50% (n=12). However there was no significance difference found between the two age groups (Fisher's exact test, *P* value = 0.2262). Two of the 27 samples were of unknown ages and could not be classified into age groups; neither produced positive PCR results.

Historical specimens belonging to the orders Anseriformes, Pelecaniformes and Psittaciformes did not successfully amplify the three fragments or the five fragments. However, their modern representatives were successfully amplified suggesting that the problem was due DNA damage rather than issues with primer binding. Bar one, all samples were over 80 years of age, stressing again the importance of age on PCR success. For the orders Charadriiformes and Anseriformes the last primer pair (AWCintF5/AWCintR6) posed a problem for some species. We were able to design an alternate primer pair (AWCintF6/AWCintR7) for these orders (refer to Table 1). This primer pair could have future use for other untested orders if a similar problem is encountered.

A number of previously described avian COI primers (Hebert *et al.* 2004; Kerr *et al.* 2009) designed to amplify a single fragment for modern samples from the Northern Hemisphere

species had limited success when applied to samples from the New Zealand and Antarctic avifauna (data not shown). In particular, the amplification of DNA from tube nosed seabirds (Procellariformes) and kiwi (Apterygiformes) proved to be difficult. A similar result was found for the internal primers AvMiF1 and AvMiR1 where preliminary tests on Procellariformes resulted in the amplification of multiple fragments. This difficulty could be attributed to the fact that New Zealand and Antarctica have a more diverse and larger number of endemic seabirds than other geographic regions like North America for which these avian primers were originally designed. Moreover, orders like the Apterygiformes (kiwi) are unique to New Zealand.

The successful amplification of the entire barcoding region using the newly designed primers AWCF1, AWCR6 and AWCR3, are shown in Table 3. The only previously published avian primer to have applicability to New Zealand and Antarctic birds was the COIbirdR2 primer (Kerr *et al.* 2009) when paired with our AWCF1 primer. This primer pair worked extremely well for Passerines (Table 3). The newly designed AWCF1/AWCR6 primer pair worked well for most orders of birds, with the forward primer AWCF1 proving to be universally successful at binding appropriately and the main reverse primer AWCR6 proving to be highly successful in the majority of cases. AWCR3 worked well in species for which AWCR6 did not prove successful. AWCR3 worked particularly well in Apterygiformes, where all five species of this order could only be amplified with the AWCF1/AWCR3 primer pair.

The majority of the sequences generated using the AWCF1/AWCR3/AWCR6 fell into the category of high quality as stipulated by the Barcode of Life Data System (BOLD) where mean PHRED scores were >40 (Ratnasingham & Hebert 2007).

Discussion

COI sequence variation between taxonomic groups has led to difficulties in the design of a single primer pair with universal applicability. Furthermore, in the case of historical samples, the 648bp COI barcoding region can only be recovered in smaller fragments requiring multiple primer sets leading to the proposition that primers must be customised to suit. However, the generation of taxa-specific primers is an impractical. A realistic solution to this problem is to generate a small set of primers that will work effectively on all avian species,

both historical and modern. The primers generated by us are an example of such sets (Figure 1). These primers, designed to amplify the barcode region of historical and modern samples from New Zealand and Antarctic bird species proved generally successful with only a few exceptional taxa, such as the Apterygiformes requiring specialised primers. Since these primers were designed using an alignment of sequences from a range of both Northern and Southern Hemisphere species, it is likely that they will be effective for avian orders from many geographic locations. It is important to note that there are a number other primers published that are not tested in this study which could prove to be highly useful (Lohman *et al.* 2009; Tavares & Baker 2008). However, our primers have a very broad applicability, having been tested on large number of species from 17 avian orders. All primers generated high quality sequences for the region of interest. Primers designed for modern samples had binding sites at least 80bp from the start and end of the barcode region to allow for good overlapping bi-directional sequence and to make allowance for the loss of sequence data which occurs at the beginning of each sequence read.

To maximise chances of obtaining amplifiable DNA from historical specimens we suggest sampling from the toe pad area where the risk of damage from preservatives is minimal. Specimen age should also be carefully considered and where possible, younger specimens should be selected for DNA analysis.

The 648bp barcode region for historical avian specimens can now be amplified using tagged internal primers as either three or five fragments, depending on the DNA quality of individual samples. We suggest using the set of primers containing three primer pairs since the likelihood of achieving success with the set of three primers pairs is the same as that from the set of five primer pairs. As our results demonstrate, if one set of internal primers does not prove successful the other set is unlikely to work either. In the event that not all overlapping fragments are amplified there is the added possibility of using one or more of these smaller fragments as a mini-barcode once this approach has be confirmed to work for avian taxa.

The addition of 5'-tags to each of the internal primers provides a number of advantages. Not only does it result in an increase in the read length of each sequence, thus allowing for better overlap of individual contigs, but it has been shown to greatly improve sequence quality (Binladen *et al.* 2007). This is crucial for historical samples where DNA damage is frequent. The 5'-tags also allow for large-scale sequencing as well as being more cost effective, as only

two primers (forward and reverse tags) are required instead of the many individual forward and reverse primers.

The disadvantage, however, is that the processes of DNA extraction, PCR amplification and sequencing from historical specimens can be taxing and time consuming. Thus, modern specimens should be utilised for barcoding purposes wherever possible. We suggest using DNA barcodes generated from historical samples as a way of adding to an already existing database where there is a lack of their modern counterparts or for cases of rare and endangered species where sample collection is problematic.

There is now an onus upon museums and other institutions to ensure the storage of well preserved vouchered samples of as many species as possible, from which, high quality DNA can be extracted. The maintenance of such collections is increasingly being adopted around the world. However, until such time as these collections become comprehensive and for rare or now extinct species, study skins and bones provide the only vouchered source of DNA. While that is the case, there is a need for methods, such as those outlined above, to provide a means of generating DNA barcodes.

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Fig. 1 A diagrammatic representation of the COI barcoding region used for birds with the relative position of the primers (triangles) indicated for amplifying historical and modern samples. The three black triangles and the two black bars indicate the combination of primers used and DNA fragments generated for modern samples. Similarly, the grey or white triangles and the grey or white bars represent the primer combinations and resulting DNA fragments generated for historical samples. The position of each primer and the amplified DNA fragments are given relative to the chicken mitochondrial genome.

References

Arctander P, Fjeldsa J (1994) Avian tissue collections for DNA analysis. ***Ibis*** **136, 359-360.**

Baker AJ, Tavares ES, Elbourne RF (2009) Countering criticisms of single mitochondrial DNA gene barcoding in birds. ***Molecular Ecology Resources*** **9, 257-268.**

Binladen J, Thomas M, Gilbert P, Campos PF, Eska W (2007) 5'-Tailed sequencing primers improve sequencing quality of PCR products. ***BioTechniques*** **42, 174-176.**

Evans T (2007) DNA damage. ***NEB expressions*** **2.1, 1-3.**

Gill FB (2007) ***Ornithology*****, 3rd edn. W.H. Freeman, New York.**

Hajibabaei M, Singer GAC, Hebert PDN, Hickey DA (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. ***Trends in Genetics*** **23, 167-172.**

Hajibabaei M, Smith AM, Janzen DH, ***et al.*** **(2006) A minimalist barcode can identify a specimen whose DNA is degraded.** ***Molecular Ecology Notes*** **6, 959-964.**

Heather BD, Robertson HA (2005) ***The Field Guide to the Birds of New Zealand,*** **Rev. edn. Penguin Books, Auckland**

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. ***Proceedings of the Royal Society of London. Series B: Biological Sciences*** **270, 313-321.**

Hebert PDN, Ratnasingham S, de Waard JR (2003b) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. ***Proceedings of the Royal Society of London. Series B: Biological Sciences*** **270, S96-S99.**

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of Birds through DNA Barcodes. ***PLoS Biology*** **2, 1657-1663.**

Hofreiter M, Serre D, Poinar HN, Kuch M, Paabo S (2001) Ancient DNA. ***Nature Reviews Genetics*** **2, 353-359.**

Hoss M, Jaruga P, Zastawny TH, Dizdaroglu M, Paabo S (1996) DNA damage and DNA sequence retrieval from ancient tissues. ***Nucleic Acids Research*** **24, 1304-1307.**

Kerr KCR, Lijtmaer DA, Barreira AS, Hebert PDN, Tubaro PL (2009) Probing Evolutionary Patterns in Neotropical Birds through DNA Barcodes. ***PLoS ONE*** **4, e4379.**

Kerr KCR, Stoeckle MY, Dove CJ, ***et al.*** **(2007) Comprehensive DNA barcode coverage of North American birds.** ***Molecular Ecology Notes*** **7, 535-543.**

Lawrence HA, Taylor GA, Millar CD, Lambert DM (2008) High mitochondrial and nuclear genetic diversity in the world's most endangered seabird, the Chatham Island Taiko (Pterodroma magentae). ***Conservation Genetics*** **9, 1293-1301.**

Lindahl T (1993) Instability and decay of the primary structure of DNA. ***Nature*** **362, 709-715.**

Lohman DJ, Prawiradilaga DM, Meier R (2009) Improved COI barcoding primers for Southeast Asian perching birds (Aves: Passeriformes). ***Molecular Ecology Resources*** **9, 37-40.**

Millar CD, Huynen L, Subramanian S, Mohandesan E, Lambert DM (2008) New developments in ancient genomics. ***Trends in Ecology & Evolution*** **23, 386-393.**

Moritz C, Cicero C (2004) DNA Barcoding: Promise and Pitfalls. ***PLoS Biology*** **2, 1529-1529.**

Ratnasingham S, Hebert PDN (2007) bold: The Barcode of Life Data System. ***Molecular Ecology Notes*** **7, 355-364.**

Sambrook J, Fritsch EF, Maniatis T (1989) ***Molecular cloning : a laboratory manual*** **Cold Spring Harbor Laboratory Press, New York.**

Seutin GW, Bradley N, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. ***Canadian Journal of Zoology*** **69, 82-90.**

Tavares E, Baker A (2008) Single mitochondrial gene barcodes reliably identify sister-species in diverse clades of birds. ***BMC Evolutionary Biology*** **8, 81.**

Yoo H, Eah J, Kim J, ***et al.*** **(2006) DNA Barcoding Korean Birds.** ***Molecules and Cells*** **22, 323-327.**

Zimmermann J, Hajibabaei M, Blackburn D, ***et al.*** **(2008) DNA damage in preserved specimens and tissue samples: a molecular assessment.** ***Frontiers in Zoology*** **5, 18.**

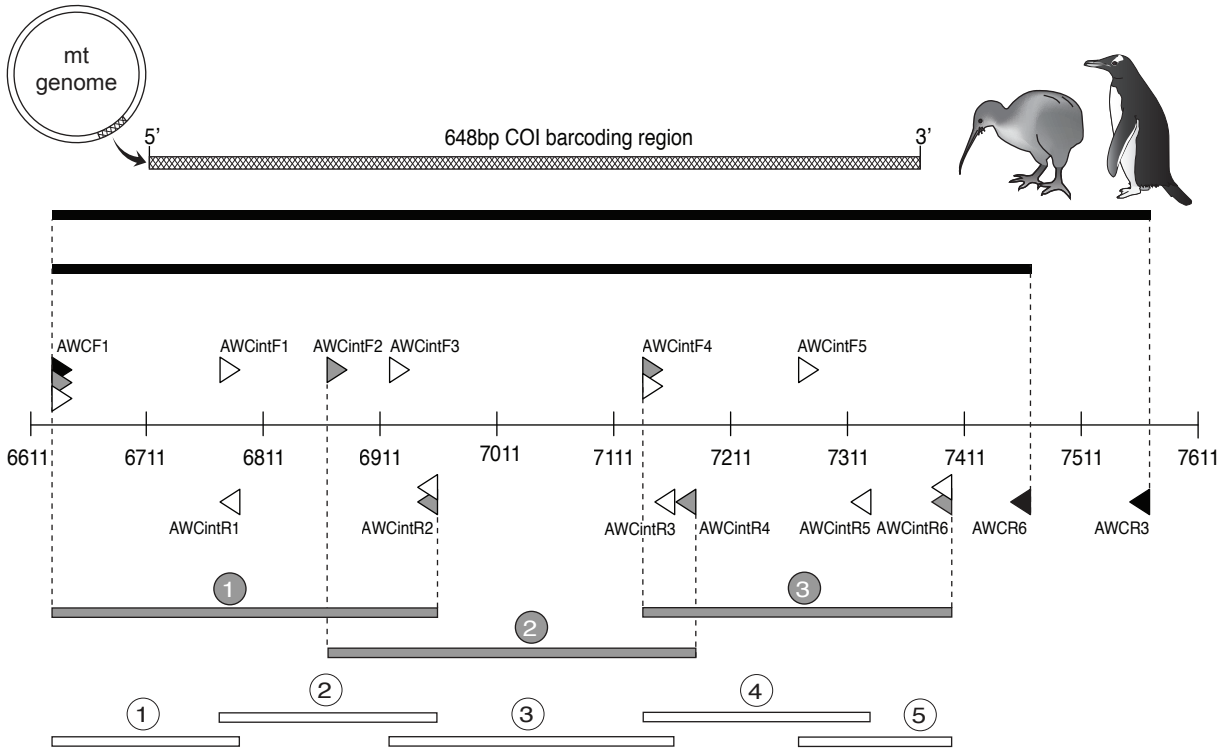
mt
genome
5'
648bp COI barcoding region
3'
AWCF1
AWCintF1
AWCintF2
AWCintF3
AWCintF4
AWCintF5
6611
6711
6811
6911
7011
7111
7211
7311
7411
7511
7611
AWCintR1
AWCintR2
AWCintR3
AWCintR4
AWCintR5
AWCintR6
AWCR6
AWCR3
1
2
3
1
2
3
4
5

Table 1 PCR primers and 5'-tags used for the amplification of the 648bp COI region as either one, three or five fragments. The position of each primer is given relative to the chicken mitochondrial sequence. The 5'-tags were only added to the individual primers when PCR amplifying and sequencing ancient samples as either three or five fragments. AWCintF6 and AWCintR7 represent alternate primer pairs used for the orders Charadriiformes and Anseriformes.

Forward Primer	Sequence 5' to 3'	Position (bp)*	Reverse Primer	Sequence 5' to 3'	Position (bp)*	Fragment Size (bp)*
	Primers for modern samples					
AWCF1	CGCYTWAACAYTCYGCCATCTTACC	6625-6649	AWCR6	ATTCCTATGTAGCCGAATGGTTCTTT	7446-7471	848
AWCF1			AWCR3	ATGCTCGGGTGTCTACGTCTAT	7542-7563	936
	First set of internal primers containing 3 primer pairs					
AWCF1			AWCintR2	ATGTTGTTTATGAGTGGGAATGCTATG		328
AWCintF2	ATCGGAGCCCCAGACATAGCATT	6912-6934	AWCintR4	TTGATGGCTGTTGTGATAAAGTTGAT	7137-7162	314
AWCintF4†	TCCTCAATCCTGGGAGCAATCAACTT	7119-7144	AWCintR6‡	GGATTAGGATGTAGACTTCTGGGTG	7371-7395	278
	Second set of internal primers containing 5 primer pairs					
AWCF1			AWCintR1	CCTGGTTGACCTAGTTCTGCTCG	6765-6787	163
AWCintF1	CCGAGCAGAACTACGTCAACC	6764-6784	AWCintR2		6926-6952	189
AWCintF3	ATAATCGGAGGCTTCGGAAACTGA	6873-6896	AWCintR3	TGGGAKAGGGCTGGTGGTTTTATGTT	7161-7186	251
AWCintF4†			AWCintR5	TGCTGGGTCGAAGAATGTGGTGTT	7299-7322	204
AWCintF5	GGCATCACCATACTACTAACAGACCG	7266-7291	AWCintR6‡			130
	5'tags for internal primers					
Ftag	AGTCGACGCTTCTAGCTT	-	Rtag	CATGCTACCTGCTACTGT	-	

†AWCintF6 – TAGGGGCAATCAACTTCATCACAAC (7129-7153) ‡AWCintR7 – ACGTRTGAGATAATTCCGAATCC (7401-7423) *basepairs

Table 2 Results of PCR success of modern and historical samples for the two primer sets designed to amplify DNA from historical specimens**.** PCR success is defined as fragments amplified (Y = amplified; N = not amplified; NT = not tested).

Order	Modern Specimens			Historical Specimens		
	3 fragments	5 fragments	No. of Species	3 fragments	5 fragments	No. of Species
Anseriformes	Y*	Y*	2	N	N	0/2
Ciconiiformes	Y	Y	2	Y	Y	2/2
Charadriiformes	Y*	Y*	2	Y*	Y*	2/4
Falconiformes	Y	Y	2	Y	Y	1/2
Gruiformes	Y	Y	2	Y	Y	1/2
Pelecaniformes	Y	Y	2	N	N	0/2
Procellariformes	Y	Y	2	Y	Y	9/10
Sphenisciformes	Y	Y	2	Y	Y	1/2
Psittaciformes	Y	Y	2	N	N	0/1
Passeriformes	Y	Y	2	NT	NT	-
Apterygiformes	Y	Y	2	NT	NT	-
Coraciiformes	Y	Y	2	NT	NT	-
Galliformes	Y	Y	2	NT	NT	-
Columbiformes	Y	Y	2	NT	NT	-
Podcipediformes	Y	Y	1	NT	NT	-

* Not all primer pairs were successful, alternate primer pairs have been supplied for these orders (see Table 1).

Table 3 PCR primer success on 17 different avian orders. The numbers represent the species of each order from which the target sequence was successfully amplified, using different combinations of PCR primers.

Order	AWCF1/R6	AWCF1/R3	AWCF1/R6 & AWCR3	AWCF1/ COIbirdR2	No. of species per order
Anseriformes	16	-	-	1	16
Apterygiformes	-	5	-	-	5
Charadriiformes	25	4	4	1	25
Ciconiiformes	9	-	-	2	9
Columbiformes	3	-	-	3	3
Coraciiformes	1	-	-	1	1
Cuculiformes	2	-	-	-	2
Falconiformes	2	-	-	1	2
Galliformes	1	-	-	1	1
Gruiformes	7	3	2	4	8
Passeriformes	17	6	1	20	20
Pelecaniformes	7	2	-	-	7
Podcipediformes	1	2	-	-	3
Procellariformes	33	5	3	5	35
Psittaciformes	4	3	2	1	5
Sphenisciformes	6	1	1	1	6
Strigiformes	1	1	1	-	1
Total	**135**	**32**	**14**	**41**	**149**

Supporting Information for online publication only

Table S1 Accession numbers of species used as part of the alignment for primer design

Scientific Name	Accession Number
Anas clypeata	AY666352
Anas platyrhynchos	AY666490
Branta canadensis	DQ019124
Arenaria interpes	AY074885
Bartramia longicauda	AY666283
Calidris alba	AY666377
Calidris fuscicollis	AY666305
Calidris mauri	AY666261
Calidris minutilla	AY666272
Larus dominicanus	AY293619
Limosa haemastica	AY666302
Pluvialis dominica	AY666317
Pluvialis squatarola	AY666202
Tringa flavipes	AY666309
Egretta novaehollandiae	DQ780878
Nycticorax caledonicus	AY666336
Eurystomus orientalis	AF407486
Chrysococcyx lucidus	AF168062
Alectoris chukar	AY666409
Callipepla californica	AY666478
Colinus virginianus	AY666347
Numida meleagris	AP005595
Phasianus colchicus	AY666332
Acanthisitta chloris	AY325307
Acridotheres tristis	AY666184
Carduelis flammea	AY666474
Corvus frugilegus	Y18522
Gymnorhina tibican	AF197868
Passer domesticus	AY666316
Pelecanus conspicillatus	DQ780883
Phaethon rubricauda	AP009043
Podiceps cristatus	AP009194
Pterodroma brevirostris	AY158678
Oceanodroma leucorhoa	AY666284
Procellaria cinerea	AP009191
Thalassarche chrysostoma	AP009193
Thalassarche melanophris	AY158677
Strigops habroptilus	AY309456
Eudyptes chrysocome	AP009189
Eudyptula minor	AF362763
Ninox nvaeseelandiae	AY309457
Apteryx australis	AF338708
Apteryx haastii	AY016010
Apteryx mantelli	AY016010

Table S2 List of historical specimens from Canterbury museum

Scientific Name	Museum Voucher ID	Collection Date (Year)
Daption capensis	Av 1212	1938
Stictocarbo punctatus	Av 649	1929
Gallirallus australis	Av 1678	1894
Falco novaeseelandiae	Av 1619	1923
Botaurus poiciloptilus	Av 17449	1958
Oceanites nereis	Av 36790	1987
Pelecanoides urinatrix	Av 512	1929
Cyanomorphus auriceps	Av 50	1895
Pterodroma macroptera	Av 1176	1928
Hymenolaimus malacorhynchos	Av 2961	1993
Anas gracilis	Av 2998	-
Charadrius obscurus	Av 2478	1922
Sterna caspia	Av 1896	1944
Eudyptes scalteri	Av 809	1923
Himantopus himantopus	Av 682	-
Micronectes giganteus	Av 2295	1939
Leucocarbo carunculatus	Av 655	1925
Egretta sacra	Av 659	1915
Falco novaeseelandiae	Av 527	1913
Porzana pusilla	Av 1654	1935
Pterodroma macroptera	Av 418	1929
Diomedea epomophora	Av 19580	1963
Oceanites nereis	Av 1302	1949
Himantopus ostralegus	Av 688	1909
Eudyptula minor	Av 822	1923
Fregata tropica	Av 502	1929
Pterodroma cervicalis	Av 1313	1914