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Ancient DNA sequences reveal unsuspected phylogenetic relationships within New Zealand wrens (Acanthisittidae)

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Abstract. Ancient DNA sequences from preserved specimens are increasingly being used for the investigation of Pacific Island ecosystems prior to the large scale modification and extinction of endemic biota associated with human colonization. However, many difficulties are associated with the use of ancient DNA sequences in studies of genetically close taxa. In this paper, these difficulties are discussed as they relate to a study involving extinct and extant members of an ancient New Zealand avian family, the New Zealand wrens (Acanthisittidae).

Sequences of the mitochondrial small ribosomal subunit RNA gene (12S) were obtained from museum specimens of several wren taxa in order to investigate their phylogenetic relationships and the taxonomic status of a rock wren (*Xenicus gilviventris*) subspecies. Limitations due to sample size and 12S sequence variability as well as the difficulties in authenticating ancient DNA sequences prevent firm conclusions but the data suggest unsuspected phylogenetic relationships exist and raise the possibility that conservation management of rock wren populations is required.

Key words. Acanthisittidae; ancient DNA; ancient populations; avian 12S DNA; contamination; New Zealand wrens.

Introduction

The use of the polymerase chain reaction (PCR[31]) to obtain ancient DNA sequences from preserved specimens is proving to be particularly useful in Oceania where relatively recent human colonization of isolated islands has caused large scale modification of endemic ecosystems[9]. Human colonization has resulted in the extinction of large numbers of morphologically unique forms, of which avian species are a particularly obvious example[3,18,25,26].

Isolation and a lack of mammalian competition has resulted in extensive morphological evolution of many Pacific Island avian species. Common modifications include gigantism (e.g. New Zealand moa), adaptive radiations (e.g. Hawaiian honeycreepers, Galapagos finches), and the loss of flight. Many island birds exhibit extensive morphological convergence as a result of their shared island habitat (e.g. gigantism and flightlessness) and can provide information about selective pressures and evolution. However, these same processes work to obscure the phylogenetic relationships of such taxa, complicating non-molecular approaches to systematics. A further difficulty is that extensive morphological modification, such as flightlessness, is commonly associated with extinction following the arrival of humans and associated mammalian predators. Molecular sequence data from preserved specimens offers a convenient method to investigate the evolutionary history of extinct taxa independent of morphological information and conflicting phylogenetic signals due to convergence. In addition, studies of ancient DNA sequences from Pacific Islands are facilated by the recent date of human colonization of Oceania[9,14] since specimens of less than 4,000 years in age can be used to analyse taxa that have become extinct due to human influence.

Naturally preserved bone and tissue samples from limestone caves, volcanic lava tubes, rock shelters, and alkali swamps, as well as material from museum collections, have begun to provide valuable molecular information about past ecosystems and evolutionary relationships of extinct taxa within Oceania[4–6,14,17]. In addition to examining extinct biota, genetic studies of surviving endemic Pacific Island taxa are also important because many are threatened due to continuing introductions of exotic biota and habitat destruction.

The human colonization of New Zealand was one of the last major colonizations within Oceania and is estimated to have taken place 800–1000 years ago[1,2]. The arrival of humans in New Zealand is associated with the extinction of many endemic avifauna[1,3], ranging in size from the flightless ratite moa (up to 270 kg in weight[6]) to the passerine New Zealand stout-legged wrens (weighing around 23–50 g[21,38]). While the extinction of the moa family (Dinornithidae) has drawn much attention[1,4] the extinction of smaller, morphologically unique avian species, such as several New Zealand wrens (including the only known flightless passerines), has drawn less interest. The New Zealand wren family (Acanthisittidae) consists of 7 species in 5 genera of which only the rifleman (*Acanthisitta chloris*) and rock wren (*Xenicus gilviventris*) are extant. Two species of stout-legged wren (*Pachyplichas yaldwyni* and *P. jagmi*) and the long-billed wren (*Dendroscansor decurvirostris*) became ex-

tinct following the arrival of Polynesians in New Zealand[21], while the Stephen's Island wren (*Traversia lyalli*) and the bush wren (*Xenicus longipes*) became extinct as a result of European activities around 1894 and 1972, respectively. The relationship between the morphologically unique New Zealand wrens and other passerines has been debated for more than a century[33] and they have been variously allied with the true songbird oscines[11,29] and suboscines[32,33]. The phylogenetic relationships of the remaining wren populations are also uncertain and have become an important conservation issue now that only two species survive. The recent extinction of the bush wren due to a lack of conservation management is a particularly poignant warning[12].

In this paper the phylogenetic relationships within the New Zealand wren family (Acanthisittidae) are investigated, and the genetic variability between wren species contrasted to that between populations of the rock wren, one of the two surviving species. Subspecies of both the rifleman and extinct bush wren are recognized in the checklist of New Zealand birds[36] but no subspecies of the rock wren has been recognized despite a description of morphological, behavioural, and habitat differences between rock wren populations south of the Hollyford River in Fiordland[10], and those in the remainder of the South Island of New Zealand. The differences were used to propose subspecies status for the southern populations (*Xenicus gilviventris rineyi*[10]) but this suggestion has never been ratified. As part of a larger study of the evolution of ancestral New Zealand avian groups, sequences of the mitochondrial ribosomal small subunit RNA gene (12S) were determined from samples obtained from museum specimens of New Zealand wrens. In this study these sequences are used to investigate the phylogenetic relationships of the New Zealand wrens and examine the genetic differences between rock wren populations south of the Hollyford River ('*X. g. rineyi*') and the remainder of the South Island ('*X. g. gilviventris*').

While the role of a museum in preserving collections must be balanced against the use of museum specimens for genetic analysis, there are many advantages in using samples from such specimens[5]. Samples obtained from museum collections permit investigations that are not possible with remains from caves or rock shelters. Records of the location, date, and sex of the collected specimens allow sequence differences observed between specimens to be analysed for geographic and temporal clines[35] and sexual dimorphism. Cave and swamp deposits of avian material in New Zealand can contain material deposited over thousands of years, sometimes with little vertical separation[38], and can potentially confuse investigation of population structure. In addition, skeletal elements of New Zealand wrens are of miniscule size (rifleman and *Xenicus* sp. range in total length from 80 to 90 mm) so naturally preserved specimens are rare and usually contain limited amounts of DNA due to erosion and chemical leaching. Museum specimens commonly offer a more concentrated source of DNA than naturally preserved material and the minute samples needed for PCR studies permit a simple sampling regime. All samples were obtained from museum collections in this study even though the rock wren and rifleman are extant, because logistical constraints prevented sampling of wild populations. Furthermore, the risk of cross-contamination (between samples) during DNA extraction is reduced if all samples are from museum specimens rather than if some samples are from modern tissue or blood.

It is important to note that ancient DNA studies are complicated by the susceptibility of the technique to contamination from external sources of DNA[28] (see also this issue). Perhaps the most significant contamination threat is DNA previously amplified in the laboratory because of the extremely high concentrations obtained via PCR amplification. Consequently, studies involving extinct taxa which are genetically close are especially challenging since contamination with previously amplified sequences, and contamination between samples, may not be easily detected. As a result, studies of ancient populations are particularly difficult due to the logistical problems involved with examining a large number of closely related samples. It is important to assess the implications of these difficulties for research in areas such as the pre-human Pacific Islands, where closely related island populations are often the subject of investigation. In this study, the molecular analysis of the New Zealand wrens is used to illustrate some of the issues that must be addressed in ancient DNA studies of populations both on Pacific Islands and elsewhere.

Methods

Two primaries and a small skin sample (approximately 3 mm^2) from the neck area were obtained from wren specimens held at the National Museum of New Zealand. Samples were obtained from one specimen each of the South Island rifleman, *A. c. chloris* (NMNZ specimen 22879), the extinct bush wren, *X. longipes variabilis* (NMNZ 11610), and rock wrens from northern and southern populations (*X. g. gilviventris*, NMNZ 12586, and *X. g. rineyi*, NMNZ 2398, respectively). The *X. g. gilviventris* specimen was collected in 1983 from Nelson, at the northern end of the range of the rock wren, while the *X. g. rineyi* specimen was collected from Fiordland in 1952. The bush wren specimen was collected from Big South Cape Island in 1955 and the rifleman from Springs Junction in 1983. The samples were removed to a laboratory where no previous studies on passerines had been carried out. A disposable scalpel was used to finely slice the bottom 2–3 mm of the feather shafts and skin samples for each specimen. Dis-

posable gloves were used at all stages to prevent cross-contamination between samples, or contamination with modern human DNA. Each combined sample was mixed with 0.75 ml of digestion buffer; 10 mM Tris-HCl pH 8.0, 2 mM EDTA, 10 mM NaCl containing 0.5 mg/ml Proteinase K (Boehringer Mannheim), 10 mg/ml dithiothreitol, and 1% SDS and shaken at 37 °C overnight. An extraction control containing no sample material was also included. All digests were extracted twice with phenol and once with chloroform:isoamyl (24:1 v/v). DNA was purified and concentrated by centrifugal dialysis in Centricon-30 disposable filtration units (Amicon, Massachusetts).

A 202 base pair (bp) fragment of the 12S gene was amplified via PCR using the primer pair:

12SK (L1941) 5′-CCTACATACCGCCGTCGCCAG-3′

$12SB_2$ (H2148) 5′-GAGGGTGACGGGCGGTATG-TACGT-3′

as described previously[5]. The numbers in parentheses refer to the position of the 3′ base of the primer in the complete chicken mitochondrial DNA sequence[8], while the letters L and H refer to the light and heavy strands respectively. $12SB_2$ is based on primer 12SB[19] and is modified to reduce competition from an internal priming site for 12SB which occurs in avian 12S sequences. PCR controls (with water substituted for DNA) and extraction controls (using an aliquot of the extraction control substituted for DNA) were amplified in parallel with all reactions, after Pääbo[28].

The sequence of the 202 bp region of each sample was determined using asymmetric PCR and chain termination sequencing as described previously[5]. The 12S fragment was amplified on two separate occasions from each specimen to test for contamination[28]. On each occasion both DNA strands were sequenced and compared to test for sequencing errors and damage-related sequence modification[27].

Results

The extraction and PCR controls were blank apart from primer dimers in all reactions, and the 202 bp region was amplified successfully in all taxa. Amplifications from the *X. g. gilviventris* extract were obtained with difficulty, and several sequencing errors were detected during analysis, suggesting that the specimen may have been treated poorly following collection. The aligned sequences of 202 bp of the 12S gene of each taxon are given in figure 1. No differences were observed between sequences obtained from separate amplifications of the same sample except as noted above. The nucleotide positions that vary between the wren sequences correspond to known variable positions in avian 12S sequences and map to loop structures in a proposed avian secondary structure (unpubl. observ.).

A distance matrix of total sequence differences between taxa is shown in the table. The two rock wren specimens are separated from the bush wren and rifleman by much larger sequence distances (5.4–6.4%) than from each other (0.5%), indicating they are most closely related. The three wren species (rifleman, bush wren and *X. gilviventris* sp.) are nearly genetically equidistant for the 202 bp region of 12S examined, with sequences that differ from 5.4–6.4%. This result is surprising given that current taxonomic classification places the bush and rock wrens in the same genus (*Xenicus*) to the exclusion of the rifleman (*Acanthisitta*[36]).

Discussion

In any ancient DNA study the first requirement is the authentification of ancient sequences. There are several stages in the experiment during which contamination may have occurred, and each must be examined.

The PCR and extract controls showed only primer dimers and no other amplification, indicating that the reactions were not inhibited but had no template DNA. This result indicates that there had not been any gross

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X.g.rineyi      TCACCTCTACTGAAAGCCCAACAGTGAACACAATAGTCCAAGCCCACTAACAAGACAGGTCAAGGTATAG
X.g.gilviventris ................................................................
Bush wren       ......T.....................................A.....................
Rifleman        ......T.G..................................G....G.................

X.g.rineyi      CCCACGGGAGGGAAGAAATGGGCTACATTCTCTAAACTAGAGCATACTGCAAGGGATGTGAAATCTTCCC
X.g.gilviventris ...................................T............................
Bush wren       ....T..............................CT..................C.........
Rifleman        ....T.....................................T....C........C.........

X.g.rineyi      CAAAAGGCGGATTTAGCAGTAAAGCGGAACAAGAATGTCCCCTTTAAACCGGCCCTAGAGC
X.g.gilviventris ................................................................
Bush wren       T.....................T.......T.GC....T.............T.......
Rifleman        ............................T.G.....................T.......
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Sequences of 202 bp of the mitochondrial 12S gene of two populations of the New Zealand rock wren (*X. g. rineyi* and *X. g. gilviventris*), aligned with the bush wren (*X. l. variabilis*) and rifleman (*A. c. chloris*). The sequence corresponds to positions 1941 to 2148 of the complete chicken mitochondrial sequence (Desjardins and Morais, 1990). Dots refer to positions homologous to the *X. g. rineyi* sequence.

Distance matrix of 202 base pair sequences of the 12S gene of the New Zealand wrens with transitions shown over transversions in the upper section of the table and uncorrected sequence distances in the lower section

	X. g. rineyi	*X. g. gilviventris*	Bush wren	Rifleman
X. g. rineyi	-	0 1	2 11	1 10
X. g. gilviventris	0.5	-	2 10	1 11
Bush wren	6.4	6.0	-	1 11
Rifleman	5.4	6.0	6.0	-

contamination of extraction or PCR amplification components. However, amplification controls have been shown to give false negatives and should not be used as sole indicators of contamination[5]. Therefore further tests must be applied to increase the chances of detecting contamination.

Several factors support the authenticity of the results. The most important indication is the different, but related, sequences gained from the four DNA extracts. This result further suggests that the extraction and PCR amplification steps were not contaminated because four different but related sources of contamination would be required, as well as differential contamination of solutions or extracts. The reproducibility of the sequence data from separate amplifications of each DNA extract also limits the possibility of contamination. Furthermore, examination of the sequences reveals phylogenetic information which is inconsistent with contamination. Taxa known to be closely related (e.g. rock wren taxa) possess the most similar sequences while all wren sequences were close but not identical to other avian 12S sequences and distant from mammalian sequences (unpubl. observ.). However due to the scarcity of material only one sample was obtained and extracted from each museum specimen so that contamination of a sample by a related museum specimen will remain a possibility until a second extraction is performed. Analysis of a second specimen of each taxon would be more conclusive, and parallel extractions using a different technique (e.g. guanidinium thiocyanate/silica[16]) would further increase the chances of detecting contaminated components.

While some workers contend that obtaining a phylogenetically compatible novel sequence is proof of the authenticity of an ancient sequence, it is important to note that there are several situations where this can be incorrect. If the sequence under analysis is heterogeneous within an organism or population under study (e.g. in mitochondria[13,20,23,30,37]), then it is possible to contaminate an ancient extract with a modern allele that is present in the laboratory but that has not yet been detected in extant taxa. Similarly, genetically related material that is present in the laboratory which has not yet been amplified and sequenced can contaminate ancient DNA studies and appear to be a novel sequence. In both situations the contaminating sequence would appear to be phylogenetically compatible. This problem can be significantly reduced if more than one specimen from a group is analysed and different, but related, DNA sequences are obtained from the specimens (e.g. ref. 4; this study). Unfortunately, this test will often be inapplicable or uninformative for studies of populations.

Because the results of this study have been examined for contamination and appear to be authentic, they will be assumed to be so, until further material can be examined. Consequently, it is appropriate to proceed with phylogenetic analysis. The rifleman, bush wren and either *X. gilviventris* sp. are separated by similar sequence distances (table) which is surprising since the rifleman is in a different genus. This discrepancy probably reflects the small amount of research that has been carried out on the phylogenetic relationships within the wrens. A limited amount of morphological analysis has been reported[24], but the morphologically unique nature of wrens has impeded research. Alternatively, the result could be erroneous due to the small number of variable sequence positions examined, although continuing studies with other mitochondrial genes indicate a similar phylogenetic relationship (unpubl. observ.). If further studies of DNA sequence data continue to show a similar genetic distance between the rifleman, rock and bush wrens it would be appropriate to reanalyse the taxonomic classification of the wrens, and consider including *Xenicus* sp. within the *Acanthisitta* genus (which has priority[33]). The sequence distance data indicate that 12S sequences may be appropriate to address this question, but that more informative sites will be required. Additional sequence information from mitochondrial and nuclear genes will be needeed to reveal the evolutionary histories of these birds.

The data appear to be inadequate to resolve the phylogenetic relationships between *X. g. gilviventris* and *X. g. rineyi* populations. The small amount of sequence difference between the two rock wren specimens (0.5%) shown in the table could be a result of stochastic variation, or partitioning of mitochondrial lineages due to different evolutionary paths for the populations. Since the section of 12S sequenced in this study is known to be one of the slowest evolving regions of the vertebrate

mitochondrial genome[22,34] even this small degree of genetic differentiation prompts investigation into the phylogenetic distinctiveness of these species.

To determine the taxonomic implications of the single transition between the rock wren specimens, the data were compared to homologous 12S sequences of other avian species. Sequences of the little and great spotted kiwi (*Apteryx oweni* and *A. haasti* respectively) show only one difference over this region[4] and different moa species of the genus *Dinornis* show no differences over this region (unpubl. observ.). These comparisons demonstrate the sequence distance observed between the *X. g. gilviventris* and *X. g. rineyi* specimens, although small, is comparable to distances observed between separate species in other avian families.

Alternatively, it is possible that the two rock wren specimens possess different mitochondrial haplotypes but are part of an interbreeding population. However, conspecific 12S sequence variability in vertebrates is thought to be extremely low due to the conserved nature and slow evolution of the gene. Studies of mammalian taxa support this view[15]. Eight specimens of New Zealand brown kiwi (*Apteryx australis*) from scattered populations in the North Island and part of the South Island show no variation in homologous sequences of the 202 bp 12S sequence used in this study (unpubl. observ.). Brown kiwi populations from the extreme south of New Zealand do show limited sequence variation but are thought to be a separate species (C. Daugherty, A. Baker pers. comm.). Sequences from three specimens of the extinct moa species *Anomalopteryx didiformis*, and three specimens of *Megalapteryx didinus* which range considerably in deposition date and geographic location also show no variation within the 202 bp region (unpubl. observ.). Because no variation is observed in the 202 bp region of 12S examined within other avian species, the sequence differences observed between the *X. g. gilviventris* and *X. g. rineyi* specimens are compatible with some period of genetic separation between the populations. However, 12S haplotype heterogeneity remains a strong possibility until more samples of both populations are examined. The sex of the wren specimens would not contribute to the observed 12S haplotype diversity because vertebrate offspring of either sex inherit the same (maternal) mitochondrial genome.

Temporal or geographic clines could also explain the sequence differences between the rock wren specimens. The possibility of a temporal cline (change in haplotype frequency over time) seems unlikely due to the known conservative evolutionary rate of 12S and because the specimens were collected within 30 years. If the specimens were collected from natural preservation sites such as a swamp or rock shelter, that contained material deposited over thousands of years, then this possibility would require further investigation. The possibility of a geographic cline between *X. g. gilviventris* in the north of the South Island and *X. g. rineyi* in the south has been raised[10] and the limited flight ability and small territories of the rock wrens may favour the formation of allopatric populations and geographic clines. To test this hypothesis 12S haplotypes of geographically intermediate rock wren populations would need to be determined. As noted previously, sequence data from studies of New Zealand kiwi and moa show little evidence of geographic clines but these birds are phylogenetically and behaviourally distant from wrens. The existence of geographic clines may often confound ancient DNA population studies because geographically intermediate specimens will often be unobtainable. Therefore discretion must be used when interpreting results obtained from isolated specimens (see also ref. 7).

In order to fully resolve the phylogenetic relationships of the various *X. gilviventris* sp. an expanded sample size, range of localities, and more informative positions will be required. In addition, nuclear DNA sequences and allozymes should also be analysed to determine non-mitochondrial genetic differentiation. In conclusion, the number of individuals sampled and the length and variability of sequence examined are too small to determine the taxonomic status of *X. g. rineyi*. While the small sequence distance observed between 12S sequences of *X. g. gilviventris* and *X. g. rineyi* are similar in magnitude to those observed between other avian species, the possibilities of sequence heterogeneity, geographic clines, and contamination must be examined further before firm conclusions can be made. This preliminary study indicates that further investigation of the taxonomic status of *X. gilviventris* is warranted in order to develop appropriate conservation management strategies.

The previously unsuspected phylogenetic relationships between the rifleman, rock and bush wrens indicated by mitochondrial DNA sequences challenge the current taxonomic classification. Further sequencing and morphological studies should address the question of merging the *Acanthisitta* and *Xenicus* genera, reflecting the similar genetic distances found between the three currently recognized members.

Some of the hazards of applying ancient DNA techniques to genetically close taxa have been discussed. Many of the criteria suggested for the authentification of ancient DNA sequences in higher level phylogenetic research have been shown to be potentially inadequate for population level studies, making the latter some of the most technically demanding in the field. Unfortunately this type of study is commonly attempted by workers who are inexperienced with ancient DNA techniques, emphasising the need for critical analysis of such work. This requirement should be noted by both workers in the field and general interest journals publishing ancient DNA research.

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1 Anderson, A., Prodigious birds. Cambridge University Press, Cambridge 1989.
2 Anderson, A., The chronology of colonisation in New Zealand. Antiquity *65* (1991) 765–795.
3 Atkinson, I. A. E., and Cameron, E. K., Human influence on the terrestrial biota and biotic communities of New Zealand. Trends Ecol. Evol. *8* (1993) 447–451.
4 Cooper, A., Mourer-Chauviré, C., Chambers, G. K. von Haeseler, A., Wilson, A. C., and Pääbo, S., Independent origins of the New Zealand moas and kiwis. Proc. natl Acad. Sci. USA *89* (1992) 8741–8744.
5 Cooper, A., DNA from museum specimens, in: Ancient DNA, pp. 149–165. Eds B. Herrmann, S. Hummel. Springer-Verlag, New York 1993.
6 Cooper, A., Atkinson, I. A. E., Lee, W., and Worthy, T. H., The evolution of the moa and their effect on the flora. Trends Ecol. Evol. *8* (1994) 433–437.
7 Davis, J. I., and Nixon, K. C., Populations, genetic variation, and the delimination of phylogenetic species. Syst. Zool. *41* (1992) 421–435.
8 Desjardins, P., and Morais, R., Sequence and gene organization of the chicken mitochondrial genome: a novel gene order in higher vertebrates. J. molec. Biol. *212* (1990) 599–634.
9 Dye, T., and Steadman, D. W., Polynesian ancestors and their animal world. Am. Scient. *78* (1990) 207–215.
10 Falla, R. A., Description of a new form of New Zealand wren. Notornis *5* (1953) 142–143.
11 Feduccia, A., Morphology of the bony stapes (columella) in the Passeriformes and related groups: evolutionary implications. Univ. Kansas Mus. Natl Hist. Misc. Publ. No. 63 1975.
12 Gill, B., and Martinson, P., New Zealand's extinct birds. Random Century, Auckland 1991.
13 Grossman, L. I., Mitochondrial DNA in sickness and in health. Am. J. hum. Genet. *46* (1990) 415–417.
14 Hagelberg, E., and Clegg, J. B., Genetic polymorphism in prehistoric Pacific islands determined by analysis of ancient bone DNA. Proc. R. Soc. B *252* (1993) 163–170.
15 Honeycutt, R.L., Allard, M. W., Edwards, S. V., and Schlitter, D. A., Systematics and evolution of the family Bathyergidae, in: The Biology of the Naked Mole-rat, pp. 45–65. Eds P. W. Sherman, J. U. M. Jarvis and R. Alexander. Princeton University Press, New Jersey 1991.
16 Höss, M., and Pääbo, S., DNA extraction from Pleistocene bones by a silica-based purification method. Nucleic Acids Res. *21* (1993) 3913–3914.
17 Huq, N. L., Tseng, A., and Chapman, G. E., Partial amino acid sequences of osteocalcin from an extinct species of ratite bird. Biochem. Int. *21* (1990) 491–496.
18 James, H. F., and Olson, S. L., Descriptions of 32 new species of birds from the Hawaiian Islands: Part II, Passeriformes. Ornith. Monographs *45*, American Ornithologists' Union, Washington, D.C. 1991.
19 Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X., and Wilson, A. C., Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. natl Acad. Sci. USA *86* (1985) 6196–6200.
20 Laipis, P. J., Van de Walle, M. J., and Hauswirth, W. W., Unequal partitioning of bovine mitochondrial genotypes among siblings. Proc. natl Acad. Sci. USA *85* (1988) 8107–8110.
21 Millener, P., and Worthy, T. H., Contributions to New Zealand's Late Quaternary avifauna. II: *Dendroscansor decurvirostris*, a new genus and species of wren (Aves: Acanthisittidae). J. R. Soc. NZ *21* (1991) 179–200.
22 Mindell, D. P., and Honeycutt, R. L., Ribosomal RNA in vertebrates: Evolution and phylogenetic applications. A. Rev. Ecol. Syst. *21* (1990) 541–566.
23 Moritz, C., and Brown, W. M., Tandem duplication of D-Loop and ribosomal RNA sequences in lizard mitochondrial DNA. Science *233* (1986) 1425–1427.
24 Oliver, W. R. B., New Zealand Birds. 2nd Ed., A. H. and A. W. Reed, Wellington 1955.
25 Olson, S. L., and James, H. F., Fossil birds from the Hawaiian Islands: Evidence for wholesale extinction by man before Western contact. Science *217* (1982) 633–635.
26 Olson, S. L., James, H, F., Descriptions of 32 new species of birds from the Hawaiian Islands: Part I, Non-Passeriformes. Ornith. Monographs *44*, American Ornithologists' Union, Washington, D.C. 1991.
27 Pääbo, S., Ancient DNA: Extraction, characterization, molecular cloning, and enzymatic amplification. Proc. natl Acad. Sci. USA *86* (1989) 1939–1943.
28 Pääbo, S., Amplifying ancient DNA, in: PCR Protocols, pp. 159–166. Eds M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White. Academic Press, San Diego 1990.
29 Raikow, R. J., Hindlimb myology and evolution of the old world suboscine passerine birds (Acanthisittidae, Pittidae, Philepittidae, Eurylaimidae). Ornith. Monographs *41*, American Ornithologists' Union, Washington, D.C. 1987.
30 Rand, D. M., Endotherms, ectotherms and mitochondrial genome-size variation. J. molec. Evol. *37* (1993) 281–295.
31 Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erhlich, H. A., Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science *239* (1988) 487–491.
32 Sibley, C. G., and Ahlquist, J. E., Phylogeny and Classification of Birds. Yale University Press, London 1990.
33 Sibley, C. G., Williams, G. R., and Ahlquist, J. E., The relationships of the New Zealand wrens (Acanthisittidae) as indicated by DNA-DNA hybridisation. Notornis *29* (1982) 113–130.
34 Simon, C., Molecular systematics at the species boundary, in: Molecular Techniques in Taxonomy, pp. 37–71. Eds G. M. Hewitt, A. W. B. Johnston and J. P. W. Young. NATO ASI series H57, Springer-Verlag, Heidelberg 1991.
35 Thomas, W. K., Pääbo, S., Villablanca, F. X., and Wilson, A. C., Spatial and temporal continuity of kangaroo rat populations shown by sequencing mitochondrial DNA from museum specimens. J. molec. Evol. *31* (1990) 101–112.
36 Turbott, E. G., (Convenor). Checklist of the Birds of New Zealand (3rd edition.) Random Century, Auckland 1990.
37 Wayne, R. K., Meyer, A., Lehman, N., Van Valkenburgh, B., and Kat, P. W., Large sequence divergence among mitochondrial DNA genotypes within populations of eastern African black-backed jackals. Proc. natl Acac. Sci. USA *87* (1990) 1772–1776.
38 Worthy, T. H., and Holdaway, R. N. H., Scraps from an owl's table; Predator activity as a significant taphonomic process newly recognised from New Zealand fossil deposits. Alcheringa (*in press*).