Flightlessness and phylogeny amongst endemic rails (Aves: Rallidae) of the New Zealand region

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SUMMARY

The phylogenetic relationships of a number of flightless and volant rails have been investigated using mtDNA sequence data. The third domain of the small ribosomal subunit (12S) has been sequenced for 22 taxa, and part of the 5′ end of the cytochrome-*b* gene has been sequenced for 12 taxa. Additional sequences were obtained from outgroup taxa, two species of jacana, sarus crane, spur-winged plover and kagu. Extinct rails were investigated using DNA extracted from subfossil bones, and in cases where fresh material could not be obtained from other extant taxa, feathers and museum skins were used as sources of DNA.

Phylogenetic trees produced from these data have topologies that are, in general, consistent with data from DNA–DNA hybridization studies and recent interpretations based on morphology. *Gallinula chloropus* (moorhen) groups basally with *Fulica* (coots), *Amaurornis* (= *Megacrex*) *ineptus* falls within the *Gallirallus*/*Rallus* group, and *Gallinula* (= *Porphyrula*) *martinica* is basal to *Porphyrio* (swamphens) and should probably be placed in that genus. Subspecies of *Porphyrio porphyrio* are paraphyletic with respect to *Porphyrio mantelli* (takahe). The Northern Hemisphere *Rallus aquaticus* is basal to the south-western Pacific *Rallus* (or *Gallirallus*) group. The flightless *Rallus philippensis dieffenbachii* is close to *Rallus modestus* and distinct from the volant *Rallus philippensis*, and is evidently a seperate species. *Porzana* (crakes) appears to be more closely associated with *Porphyrio* than *Rallus*. Deep relationships among the rails remain poorly resolved. *Rhynochetos jubatus* (kagu) is closer to the cranes than the rails in this analysis.

Genetic distances between flightless rails and their volant counterparts varied considerably with observed 12S sequence distances, ranging from 0.3% (*Porphyrio porphyrio melanotus* and *P. mantelli mantelli*) to 7.6% (*Rallus modestus* and *Rallus philippensis*). This may be taken as an indication of the rapidity with which flightlessness can evolve, and of the persistence of flightless taxa. Genetic data supported the notion that flightless taxa were independently derived, sometimes from similar colonizing ancestors. The morphology of flightless rails is apparently frequently dominated by evolutionary parallelism although similarity of external appearance is not an indication of the extent of genetic divergence. In some cases taxa that are genetically close are morphologically distinct from one another (e.g. *Rallus* (*philippensis*) *dieffenbachii* and *R. modestus*), whilst some morphologically similar taxa are evidently independently derived (e.g. *Porphyio mantelli hochstetteri* and *P. m. mantelli*).

1. INTRODUCTION

The central tenet of Charles Darwin's evolutionary hypothesis is the concept of 'descent with modification', which he developed despite the extreme difficulty of observing the process. Darwin supported his theory by citing a multitude of examples testifying to the diversity and adaptation of organisms, gaining some of his greatest insights and strongest evidence from observations of island faunas (Darwin 1839, 1859). Since that time islands have remained valuable research tools for the study of endemism and processes of speciation (Grant 1981; Freed *et al.* 1987; Otte & Endler 1989; Cooper & Millener 1993). In recent years this research has benefited considerably from the development of molecular techniques which have the power to elucidate phylogeny, even in the face of morphological convergence, and to provide an approximate time-scale for speciation events (e.g. Tarr & Fleischer 1993; Thorpe *et al.* 1994). In the present study, molecular methods have been applied to the rails, a group of birds which has not gained quite the same notoriety as Darwin's finches or the Hawaiian honey-eaters as an example of species radiation, but is, nevertheless, of profound interest to evolutionary biologists.

The Rallidae have a comprehensive geographic distribution across both continental and oceanic islands (Olson 1973*a*) and a great many (more than one quarter of extant or recent extinct species) are flightless (Olson 1973*a*; Diamond 1981). Many of them have recently been brought to extinction by humans and various introduced predators which have colonized islands on which they once existed (Steadman & Olson 1985; Steadman 1995). The Pacific region was once very rich in flightless rails, as indicated by the extensive deposits of subfossil bones found in the Hawaiian archipelago (Olson & James 1991), in New Zealand (Millener 1991), and on other islands (Steadman 1986,

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1988, 1995; Wragg 1995). It is likely that further, recently extinguished species, have yet to be accounted for (Pimm *et al.* 1994). The majority of the world's extant flightless rails persist in the New Zealand region. New Zealand itself boasts two species in separate genera, and several extinct species (Olson 1975; Millener 1991).

The impact of introduced predators (including humans) on secondarily flightless avifauna is an indication that the earlier predator-free status of oceanic islands was of particular significance in the evolution of these species. The frequency with which flightlessness has evolved among rails in such circumstances, is probably a function of both the behavioural flightlessness exhibited by many volant species (Ripley 1977), and the seemingly paradoxical frequency with which these species reach distant predator-free islands (Olson 1973*a*; Ripley 1977; Olson & James 1991). It is generally assumed that flightless rails are derived from self-colonizing volant ancestors, although a panbiogeographic analysis suggested that the flightless weka (*Gallirallus australis*) and its ilk may have evolved from a common flightless ancestor (Beauchamp 1989). However, in the absence of molecular data, the self-colonization hypothesis, which explains similarities in gross morphology among such species as being the result of convergence associated with flight loss rather than being of phylogenetic significance, is less controversial.

Although gross skeletal characteristics such as relative dimensions of leg and wing bones tend to be congruent amongst flightless species, analysis of data from sufficient material can reveal significant differences between birds that have at one time been classified as the same species. For instance, differences in the relative dimensions of bones from two morphotypes of the takahe (*Porphyrio mantelli*), are consistent with the notion that these two forms were independently derived from separate (but similar) volant ancestors, rather than having diverged after flight loss (Trewick 1996). The implications of the independent origin hypothesis are considerable in that they indicate an unexpected level of evolutionary repeatability and contradict the reasonable assumption that morphologically similar, and geographically proximal 'forms' are necessarily each their closest relatives. Furthermore, it emphasizes that as a factor in speciation, the loss of flight is associated with a process of morphological parallelism rather than divergence.

In the past there has been a tendency to view the distinctiveness of flightless island endemics as indicating that those species had ancient origins that deserved recognition at the generic level (Ripley 1977). Hence, many monotypic genera have been proposed, such as *Notornis* for the takahe (Owen 1848), *Cabalus* for the small Chatham rail (Hutton 1874), and *Nesophalaris* for the flightless Chatham coot (Brodkrob & Dawson 1962). Subsequently, some of these genera came to include additional flightless species from separate islands (e.g. *Notornis alba* on Lord Howe, Rothschild 1907) even though this required either a vicariant origin of members of such genera, or that the genera were paraphyletic with respect to their volant counterparts. Recent treatments have tended to resolve this problem by suggesting that, despite sometimes profound differences in gross morphology, flightless endemics are probably recently derived, and monophyletic with their volant counterparts (Olson 1973*b*). In the case of *Notornis*, Greenway (1967) stated that any similarity between geographically isolated taxa may have resulted from 'accidental parallelism in evolution'.

Recognizing the possible consequences of morphological convergence in phylogenetic analysis I have taken a molecular approach to examine the relationships of extant and extinct flightless rails and their volant counterparts. This has additional merit in that it is possible to obtain genetic markers from rare species through the use of museum material, and from recently extinct species through the use of subfossil bones as sources of DNA. Genetic evidence for the relationships of some rails has already been provided by DNA–DNA hybridization studies (Sibley & Ahlquist 1990). Mitochondrial DNA (mtDNA) sequence analysis, the method employed in the present study, has been used widely in phylogenetic reconstructions of various organisms including many other avian groups (Edwards *et al.* 1991; Quinn *et al.* 1991; Cooper *et al.* 1992; Krajewski & Fetzner 1994), but has not previously been applied to rails. This paper therefore describes the use of new DNA sequences to examine the phylogeography at the species level, of morphologically similar flightless rails and their volant relatives occupying islands in the New Zealand region.

2. METHODS

(*a*) *Materials*

The biological nomenclature used in this paper follows Ripley (1977) for the rails. Despite the lack of any formal character analysis and the rather enigmatic placement of some taxa it is a well illustrated and convenient text. Its use in the present work does not indicate an endorsement of that classification, and where helpful I have indicated the taxonomy proposed by other authors, such as Olson (1973*a*). Many of the taxa used in this analysis are flightless species found in the New Zealand region. Related volant species and other representatives from a wider geographic and phylogenetic area have also been included to scale the data set (figure 1). Outgroup taxa used include the kagu (*Rhynochetos jubatus*) and sarus crane (*Grus antigone*), representing a possible sister family of the rails in the Gruiformes (see Sibley & Ahlquist (1990) for a review of taxonomy); and the comb-crested jacana (*Irediparra gallinacea*) and the wattled jacana (*Jacana jacana*) which have several features (fleshy cranial ornamentation, and morphology associated with aquatic habit) in common with members of the Rallidae; and the Australian spurwing plover (*Vanellus miles novaehollandiae*). All of the latter species belong to the order Ciconiformes according to Sibley & Ahlquist (1990). Nomenclature of non-rails follows Howard & Moore (1980).

Where possible, fresh tissues (heart, liver or blood) were used as sources of DNA. Where such material

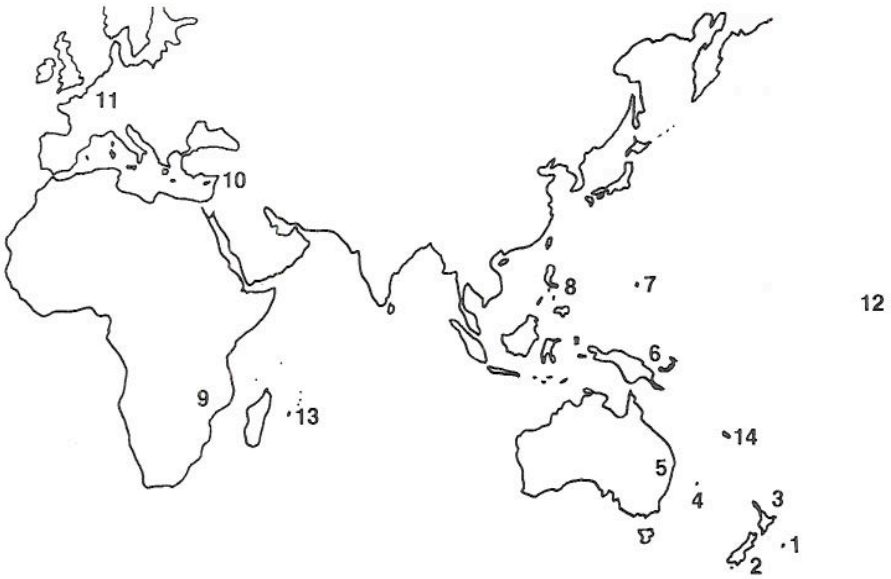

Figure 1. Locations of geographic areas referred to in text and on other figures, corresponding to regions occupied by the rail taxa used in the study. 1, Chatham Islands; 2, South Island New Zealand; 3, North Island New Zealand; 4, Lord Howe Island; 5, Eastern Australia; 6, New Guinea; 7, Guam; 8, Philippines; 9, southern Africa; 10, Turkey; 11, Europe; 12, central and southern America; 13, Mauritius and Reunion; 14, New Caledonia. The precise origins of specimens used are given in the text.

could not be obtained (a majority of cases), tissue samples were preserved feathers or skins. Skin was generally taken from the underside of the foot (using a sterile scalpel) in order to retain the morphological integrity of specimens. For species represented by quaternary fossils only, bones that had accumulated in dry, undisturbed conditions were selected for DNA extraction. Most species were represented by a single specimen, except for the polytypic species *Porphyrio porphyrio* and *Rallus philippensis*. At least two tissue samples were taken from each specimen and analysed until unambiguous sequences were produced.

Voucher information. Takahe (*Porphyrio m. hochstetteri*) feather and skin samples came from specimens later prepared as skeletons, and feathers from live individuals. The feather sample from *Fulica atra* was taken from an individual at Wanganui (New Zealand) which was subsequently re-released. Feathers of *Grus antigone* and *Porphyrio p. madagascariensis* were collected from live individuals at Le Jardin aux Oiseaux, France. The cytochrome-*b* sequences for *Grus antigone* came from Carey Krajewski. Abbreviations: NMNZ, National Museum of New Zealand (FTC-frozen tissue collection); WO, Waitomo Caves Museum; BMNH, British Museum (Natural History), Tring; LSUMNS, Louisianna State University Museum of Natural Science; USNM, United States National Museum (Smithsonian Institution); TM, Transvaal Museum; LIVCM, National Museums and Galleries on Merseyside; MV, Museum of Victoria, Australia; CC, Chris Collet; SE, Stephen Emlen; SAT, the author; † indicates extinct species. Species (common name), tissue type, source registration (origin of specimen): *Amaurornis ineptus* (New Guinea flightless rail), foot skin, BMNH 1911.12.20.337 (Setakwa River, New Guinea); *Eulabeornis cajaneus* (graynecked wood rail), liver, LSUMNS B-10828 (Rio Shesha, Peru); *Fulica atra australis* (coot), feather, SAT (Wanganui, NZ); *F. chathamensis chathamensis* (giant coot)†, foss. bone, NMNZ S-27463#49 (Te ana a Moe, Chatham Is.); *F. c. prisca* (giant coot)†, foss. bone, NMNZ DM 379 (Castle Rocks, NZ); *Gallinula chloropus* (moorhen), foot skin, LIVCM 1983.407 (Essex, UK); *G. martinica* (purple gallinule), skin, LIVCM 1984.2.371 (Tristan da Cunha); *G. martinica* (purple gallinule), liver, LSUMNS B-20812 (Louisiana, USA); *Gallirallus australis* (weka), blood, NMNZ (FTC) L30653 (Kapiti Is., NZ); *G. a. hectori* (buff weka), liver, SAT (Tuku Res., Chatham Is.); *Irediparra gallinacaea*, liver, MV E001 (Port Morsby, Papua New Guinea); *Jacana jacana*, blood, SE (Panama); *Porphyrio mantelli hochstetteri* (South Island takahe)†, feather/skin, SAT-NMNZ (Fiordland, NZ); *P. m. mantelli* (North Island takahe)†, foss. bone, NMNZ C.138 (Coonoor, NZ); *P. m. mantelli* (North Island takahe)†, foss. bone, NMNZ M.1/136 (Martinborough, NZ); *P. p. madagascariensis* (swamphen), feather, SAT (Upie, France); *P. p. madagascariensis* (swamphen), liver, TM 61998 (Ventersdorp, Transvaal, S. Africa); *P. p. melanotus* (swamphen, pukeko), feather, SAT (Kiringe creek, Chatham Is.); *P. p. melanotus* (swamphen, pukeko), liver, SAT (Hawkes Bay, NZ); *P. p. melanotus* (swamphen, pukeko), liver, MV B64 (Australia); *P. p. pulverulentus* (swamphen), foot skin, USNM 578176 (Lake Mainit, Mindanao Is., Philippines); *P. p. pulverulentus* (swamphen), foot skin, USNM 578177 (Lake Buluan, Mindanao Is., Philippines); *P. p. seistanicus* (swamphen), foot skin, BMNH 1965.M.2494 (Hatay, Turkey); *Porzana pusilla affinis* (tiny crake), feather, NMNZ NM-24061 (Whangamarino, NZ); *P. tabuensis* (spotless crake), feather, NMNZ NM-24401 (Waikanae, NZ); *Rallina castaneiceps* (chestnut-headed crake), liver, LSUMNS B-9104 (Cobija, Bolivia); *Rallus aquaticus* (water rail), foot skin, LIVCM 1983.352 (Bardsey Is., Wales); *R. modestus* (Chatham

Table 1. *PCR primers and conditions*

12S rRNA primer sequences.	
L1753 (12SA)	5′-CAAACTGGGATTAGATACCCCACTAT-3
H2171 ($12SB_2$)	5′-GAGGGTGACGGGCGGTATGTACGT-3′
L1873 (12SE)	5′-CCCAACCTAGAGGAGCCTGTTC-3′
L1999 (12SJ)	5′-CCCCCGCTAACAAGACAGGT-3′
H2023 ($12SF_2$)	5′-GGAAGTTAGGTCAAGGTGTA-3′
H1892 (12SG)	5′-GGCAAGAGATGGTCGGGTGTA-3′
Cytochrome-*b* primer sequences.	
L14841	5′-AAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3′
H15156	5′-AAACTGCAGCCCCTCAGAATGATATTT-3′

PCR conditions for 12S amplification were 35–45 cycles of the denaturation (D) (94 °C, 40 s), primer annealing (A) (55 °C, 40 s) and polymerase extension (E) (72 °C, 60 s). Cytochrome-*b* amplification used a ramped program of 1 cycle; D(94 °C, 60 s)- A(48 °C, 40 s)- E(72 °C, 60 s), 1 cycle; D(94 °C, 40 s)- A(50 °C, 40 s)- E(72 °C, 60 s), and 35–40 cycles; D(94 °C, 40 s), A(52 °C, 40 s), E(72 °C, 60 s).

Codes for 12S and cytochrome-*b* primers refer to the numerical position of the 3′ base of the primer on the light (L) and heavy (H) strands of the complete chicken mitochondrial DNA sequence (Desjardins & Morais 1990), and the complete human mitochondrial DNA sequence (Anderson *et al.* 1990), respectively. 12SA and $12SB_2$ described in Cooper (1994), other 12S primers in Cooper *et al.* (1992). L14841 as described by Thomas *et al.* (1989), and H15156 (G. K. Chambers, personal communication) were derived from H15149 in Kocher *et al.* (1989).

rail)†, foss. bone, NMNZ S-27480#38 (Te ana a Moe, Chatham Is.); *R. owstoni* (guam rail), liver, LSUMNS B-20078 (Guam); *R. philippensis assimilis* (banded rail), feather, NMNZ, NM-23996 (Mt Bruce, NZ); *R. p. australis* (banded rail), liver, MV, D428 (Kingston, Austalia); *R. p. dieffenbachii* (Dieffenbach's rail)†, foss. bone, NMNZ S-30120#18E/91 (Te ana a Moe, Chatham Is.); *R. sylvestris* (Lord Howe wood rail), DNA, CC #41 (Lord Howe Island); *Rhynochetos jubatus* (kagu), flesh, SAT, GH 24 & 30 (New Caledonia); *Vanellus miles novaehollandiae* (spurwing plover), feather, SAT (nr Whangamerino, Chatham Is.).

(*b*) *Laboratory procedures*

DNA was extracted from soft tissues using a method of Proteinase K digestion followed by extraction with phenol and chloroform (Sambrook *et al.* 1989). Soft tissue samples (alcohol-preserved liver, blood, fresh feathers) were fragmented where necessary using a sterile scalpel blade and treated with 0.5 ml of a buffered Proteinase K solution previously prepared and stored frozen at −20 °C in 1.5 ml microcentrifuge tubes (final concentrations: 10 mM Tris-HCl pH 8.0, 2 mM EDTA, 10 mM NaCl, 500 μg ml^{-1} Proteinase K, 10 mg ml^{-1} dithiothreitol, 1% SDS). Samples were turned continuously at 50 °C for up to 24 h (see also Leeton *et al.* 1992). Alternatively, 2 μl blood samples were treated with 5% Chelex 100® and lysed by boiling (Walsh *et al.* 1991). Samples removed from museum study skins were cleaned and pre-softened in several changes of sterile distilled water (or a 5% chelex solution) at 50 °C for 24 h, and then treated with Proteinase K as above.

DNA was extracted from quaternary fossil bones using a similar Proteinase K digestion method as above, after initial decalcification with EDTA (Hagelberg *at al.* 1989; Hagelberg & Clegg 1991; Cooper 1994). Fossil bone was sampled using a hacksaw blade except in the case of very small elements which were used whole. The external surface of samples was first cleaned with sand paper and the samples ground using a small coffee grinder reserved explicitly for this purpose, and thoroughly cleaned between samples. Approximately 1 g of bone powder was used, and this treated with about 20 ml of 0.5 M EDTA (pH 8.0). The mixture was turned at room temperature for 12–24 h and then centrifuged at 3000 rpm for 5 min to separate solid matter, which was then treated with Proteinase K digestion mix followed by two extractions with an equal volume of glass-distilled grade phenol and one of chloroform/isoamyl alcohol (24:1). All fossil bone extractions were undertaken at a separate site from treatment of modern DNA sources and amplification procedures to avoid cross-contamination. Parallel blank extraction and PCR controls were used to detect reagent contamination.

Purified DNA was either used directly following extraction with phenol or after concentration by ethanol precipitation, or by use of Millipore® microconcentrator tubes (Leeton *et al.* 1992). Segments of the mitochondrial genes, cytochrome-*b* and 12S ribosomal RNA (12S) were amplified using the polymerase chain reaction (PCR, Saiki *et al.* 1988). The external 12S primers used amplify the third domain of the small ribosomal subunit, approximately 400 bp. The two external cytochrome-*b* primers amplify a fragment of around 300 bp (see table 1 for details). PCR-catalysed target amplification was performed in 25 μl volumes incorporating 1.5 mM $MgCl_2$, 0.575 units *Thermus flavus* (Tfl) DNA polymerase (Epicentre Tech.), and each primer at a concentration of 1 μM. The thermal cycling programme used a Perkin-Elmer Cetus DNA Thermocycler, Model 480, and began with 3 min at 94 °C and was terminated with 10 min at 72 °C, see table 1 for conditions.

PCR products were purified by electrophoresis on 2% low-melting point agarose (LMP) gels run in Tris-Acetate (TA) buffer (TAE buffer, Maniatis *et al.* (1982), without EDTA) and stained with ethidium

bromide. Initially, asymmetric PCR amplification was undertaken using a 1 µl aliquot of a solution made from an agarose gel slice containing double stranded PCR product, which had been suspended in 100 µl of distilled water at 65 °C. Asymmetric amplification reactions used the same thermal cycle parameters as above but contained one primer at lower concentration, optimum ratios used were between 1:10 and 1:50 (Gyllensten & Erlich 1988; McCabe 1990). The single stranded DNA products obtained were sequenced using the dideoxynucleotide sequencing method (Sanger *et al.* 1977) with the Sequenase kit version 2.0 (United States Biochemicals; Sambrook *et al.* 1989). Subsequently, a more rapid method that did not require the production of single stranded templates was employed. Agarose plugs containing the double stranded target product and a minimum of agarose were cut from purifying gels made from 2% LMP agarose in TA buffer. These were stored at −20 °C and used in the sequencing method described by Trewick & Dearden (1994).

(*c*) *Data analysis*

DNA sequences were read from autoradiographs and manually aligned using the computer package ESEE (Cabot & Beckenbach 1989) with other overlapping sequences from the same DNA samples, and combined. Unambiguous consensus sequences for all individuals were then aligned. Alignments of 12S sequences were facilitated by reference to the secondary structure configuration of Hickson *et al.* (1996). This was particularly helpful with the alignment of insertion/deletion regions. The 12S data set analysed was slightly reduced to exclude sites with doubtful homology associated with insertion/deletion events which are frequent in certain regions of the 12S molecule (Neefs *et al.* 1990). Nucleotide sequences of the protein-coding cytochrome-*b* gene were translated into amino acid sequences, and compared with one another and with the structural hypothesis for cytochrome-*b*, in order to confirm substitutional consistency and thus mitochondrial origin of the nucleotide sequences obtained (Kocher *et al.* 1989).

Phylogenetic analysis utilized the computer software packages PAUP 3.0 (Swofford 1993), for parsimony analysis, MEGA 1.02 (Kumar *et al.* 1993) and SplitsTree 1.0.1 (Huson & Wetzel 1994) for distance methods, and Hadtree (Penny *et al.* 1993) for spectral methods (Lento *et al.* 1995). Maximum likelihood (ML) methods were performed with DNAML and DNAMLK 3.51c in PHYLIP (Felsenstein 1993) and PUZZLE 2.5.1 (Strimmer & von Haeseler 1996). Unweighted parsimony analysis utilized heuristic (for large numbers of taxa) and branch and bound searches. Neighbour-joining (NJ) analysis and SplitsTree used distance matrices containing numbers of differences (observed distance) or distances corrected for multiple hits (Kimura 1980, 1981). In cases where all methods gave a tree of equivalent topology those presented are based on uncorrected distance measures in accordance with the recommendations in Kumar *et al.* (1993).

Support for NJ trees was assessed by the calculation of bootstrap confidence levels using MEGA. SplitsTree, which uses a split decomposition algorithm (Bandelt & Dress 1992), shows relative support for alternative nodes by giving polygonal rather than simple linear edges in cases where conflicting signals are encountered. Noise in the data set, resulting from multiple hits, is indicated by the 'fit' value. Fit is calculated from what is termed the 'split prime residue' (Huson & Wetzel 1994), such that a higher value (up to 100%) accords with a higher proportion of the signal being represented by the output tree (see Lockhart *et al.* (1995) and McLenachan *et al.* (1996) for examples of the use of SplitsTree).

Phylogenetic trees presented are derived from MEGA and SplitsTree, with annotations made in MacDraw 1.9. Analysis of 12S data for closely related taxa (*Porphyrio*, *Rallus*) used a data set that retained some variable sites located in hyper-variable regions that could not be unambiguously aligned across all taxa, but for which it was possible to verify the homology in more closely related taxa. Sequence data presented here have been deposited with GenBank under accession numbers U77138–U77177. Aligned 12S sequences are given in the appendices.

3. RESULTS

The 246 bp segment from the cytochrome-*b* gene of 12 species of rails, contains 67 (27%) variable sites, 39 (15.8%) of which are phylogenetically informative. This fragment corresponds to positions 15022–15268 in the published chicken mtDNA sequence (Desjardins & Morais 1990). The 12S sequence haplotypes of approximately 400 bp were obtained from 22 species of rails, have a total of 117 (30%) variable sites with 82 (21%) informative. Additional 12S sequences were produced for kagu, sarus crane, spurwing plover and two species of jacana. Sequences were not obtained for both genes from all taxa, owing principally to the poor utility of cytochrome-*b* primers for PCR amplification of 'ancient DNA' (i.e. that extracted from subfossil bones and museum skins) encountered in this study. Tables 2 and 3 show pairwise comparisons of numbers of nucleotide substitutions and the per cent sequence divergence for cytochrome-*b* and 12S data, respectively.

Base composition and substitution characteristics of cytochrome-*b* sequences from rails conform to those encountered among other birds (Kocher *et al.* 1989; Edwards *et al.* 1991; Johnson & Cicero 1991; Krajewski & Fetzner 1994). Substitutions are concentrated in the third codon position (85%), with fewer, 13.5% and 1.5%, at the first and second positions respectively. Overall nucleotide composition of cytochrome-*b* sequences are similar among taxa, with a light-strand bias towards cytosine derived principally from an excess at third codon positions. A strong bias against guanine and thymine at third codon positions is also evident. The 12S gene fragment has a 1:1 AT:GC ratio with a slight bias toward adenine and cytosine on the light strand.

In pairwise comparisons of nucleotide substitutions over 246 bp cytochrome-*b* sequence positions, 3–9

Table 2. *Matrix of numbers of transition and transversion substitutions (lower) and observed per cent nucleotide divergence (upper) from pairwise comparison of 246 bp cytochrome-*b *sequences from rails and sarus crane* (Grus antigone)

(Boxes A (purple swamphens) and B (brown rails) enclose groups with flightless taxa; probable congenerics within light border, more closely related taxa groups with flightless taxa; probable congenerics within heavy border. Flightless taxa are indicated by the standing bird icon beside taxon names.)

		1	2	3	4	5	6	7	8	9	10	11	12	13
Gallinula martinica	1	A	11.0	11.8	10.6	16.7	15.0	14.6	13.4	11.4	12.6	14.2	13.4	12.6
Porphyrio mantelli hochstetteri	2	17\10		3.7	3.3	11.0	11.0	13.0	11.8	10.2	11.0	12.6	13.4	14.2
Porphyrio p. melanotus	3	19\10	9\0		2.0	11.8	11.4	12.6	11.4	10.6	10.6	11.4	12.2	14.6
Porphyrio p. pulverulentus	4	15\11	7\1	4\1		11.0	11.4	11.8	11.0	9.3	10.2	11.8	12.6	13.4
Porzana tabuensis	5	25\16	17\10	19\10	16\11		9.3	13.0	12.6	11.8	12.6	14.6	12.6	18.7
Porzana pusilla affinis	6	19\18	17\10	18\10	17\11	19\4		13.0	10.6	11.4	10.6	13.4	12.6	14.2
Rallus aquaticus aquaticus	7	20\16	20\12	19\12	16\13	20\12	20\12	B	8.9	8.5	9.3	11.4	9.3	15.0
Rallus philippensis australis/assimilis	8	17\16	17\12	16\12	14\13	17\14	10\16	14\8		2.4	1.2	6.5	6.5	14.6
Rallus owstoni	9	14\14	15\10	16\10	12\11	15\14	12\16	14\7	5\1		3.7	7.3	6.9	15.0
Rallus sylvestris	10	16\15	16\11	15\11	13\12	18\13	11\15	15\8	3\0	8\1		6.9	6.9	13.0
Amaurornis inepta	11	21\14	21\10	18\10	18\11	22\14	17\16	20\8	12\4	13\5	13\4		5.7	14.6
Gallirallus australis	12	19\14	23\10	20\10	20\11	19\12	17\14	17\6	14\2	15\2	15\2	12\2		16.3
Grus antigone	13	13\18	19\16	20\16	16\17	28\18	21\14	21\16	20\16	19\18	15\17	18\18	24\16	

transitions (TS) and 0 and 1 transversions (TV) were found between closely related species (*Rallus sylvestris* : *R. philippensis*, *Porphyrio p. melanotus* : *P. m. hochstetteri*). Between more distant congenerics (*R. philippensis* : *R. aquaticus*) TS and TV were 14 and eight respectively (8.9% divergence). Between genera, TS and TV reached a maximum of 25 and 18 respectively. Between the crane and the rails, TS were generally not much more frequent than this (the highest value being 20), and TV ranged between 14 and 18 with a maximum divergence of 18.7%, indicating that a relatively high degree of cytochrome-*b* sequence divergence exists within the rails themselves, and that saturation was likely to influence phylogenetic reconstruction at basal nodes. *Gallinula martinica* showed particularly high divergence from other rails in the cytochrome-*b* gene (table 2), but did not show abnormal amino acid replacement (Kocher *et al.* 1989).

The 12S sequences (388 homologous sites) were relatively less diverged from one another than were cytochrome-*b* sequences and this is consistent with evidence from other studies that suggest the 12S gene evolves less rapidly than cytochrome-*b* (Thomas *et al.* 1989; Lento *et al.* 1995). The 12S sequences had between one and 16 transitions and 0 and three transversions between subspecies of *Porphyrio* (0.3–5% divergence, table 3). The number of TS and TV observed in comparisons of subspecies of *Porphyrio* were on the whole greater than amongst currently recognized species of other rail genera such as *Rallus*. The highest number of TS observed in comparisons between the rails was 43 (TS values observed within genera were generally greater than 20), the highest number of TV within the rails was 15. Between rails and the crane there were 26 to 36 TS and 15 to 24 TV (10.7–14.1% divergence, table 3). Subspecies of *Gallirallus australis*, *Fulica chathamensis* and *Rallus philippensis* (with the exception of *R. p. dieffenbachii*) could not be distinguished from their 12S gene sequences. This is in contrast to the situation encountered with the 'subspecies' of *Porphyrio porphyrio*. A similar pattern was observed in the comparison of cytochrome-*b* sequences, indicating that the subspecies of *Gallirallus australis* and *Rallus philippensis* (*australis* and *affinis*) are of more recent origin than *Porphyrio porphyrio*. Minimum distances encountered between flightless species and volant relatives were 0.3% in the 12S data, and 1.2% in cytochrome-*b* sequences.

Phylogenetic reconstruction using neighbour-joining (NJ) of observed distances for the 12S data yielded the tree shown in figure 2. Taxa generally fall into generic groups in accordance with the traditional taxonomy, but deeper internal edges are not well supported (bootstrap confidence levels below 50%). These could, for practical purposes, be considered as polytomies. However, the topology of this tree is compatible with that given by DNA–DNA hybridization (Sibley & Ahlquist 1990). Using the same data, SplitsTree produces a tree of the same topology, but collapses nodes that are weakly supported in NJ forming a 'starfish' pattern, and the SplitsTree fit value for this large number of taxa is only 44%. Parsimony analysis in PAUP, using the heuristic search option in order to accommodate the large number of taxa, returned a topologically similar tree (not shown), with the *Porzana* group more basal and *Amaurornis* and *Gallirallus australis* in a discrete clade. Hadamard distance analysis gave the same groupings and associations as NJ. ML methods (DNAML and DNAMLK in PHYLIP, and PUZZLE) also produced topologically similar trees with the exception that the position of *R. casteneiceps* proved inconsistent, although always basal amongst the rails in this analysis. More importantly, both PUZZLE and DNAML placed *G. australis* below *R. aquaticus* in that clade, although DNAMLK (which assumes a molecular clock) gives the same ordering of these three as NJ (figure 2). This suggests that the arrangement of *Amaurornis* (= *Megacrex*) *ineptus* and *Gallirallus australis* (weka) among the other flightless brown rails is equivocal and requires the inclusion of further congenerics to be resolved. The purple gallinule (*Gallinula martinica*) is apparently misplaced, but this reflects Ripleys (1977) inclusion of this species with the moorhens. It is evidently better referred to *Porphyrula*

Table 3. *Matrix of numbers of transition and transversion substitutions (lower) and observed per cent nucleotide divergence (upper) from pairwise comparison of 388 bp 12S sequences from rails and sarus crane* (Grus antigone)

(Boxes A (purple swamphens) and B (brown rails) and C (coots) enclose groups with flightless taxa; probable congenerics within light border, more closely related taxa within heavy border. Flightless taxa are indicated by the standing bird icon beside taxon names.)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Gallinula martinica	1	A	5.2	6.0	7.3	7.3	6.5	6.0	6.3	10.2	11.0	10.5	10.8	10.8	11.0	11.6	11.8	10.3	9.7	9.7	10.0	11.2	10.7	11.0	13.9
Porphyrio mantelli hochstetteri	2	15\4		1.3	3.1	2.9	3.7	1.6	1.3	10.4	10.2	10.7	10.7	10.2	11.5	11.0	11.3	9.2	8.9	10.4	9.9	9.6	9.6	9.6	12.3
Porphyrio porphyrio melanotus	3	19\4	6\0		2.1	1.8	3.7	0.5	0.3	9.9	10.2	11.0	11.5	11.0	11.8	11.8	12.0	10.0	9.7	11.2	9.7	9.4	8.9	9.9	12.0
Porphyrio p. pulverulentus (a)	4	23\5	11\1	7\1		0.3	5.0	1.6	1.8	10.7	9.9	12.8	12.3	11.8	13.1	12.6	12.8	11.3	10.5	13.1	11.0	10.7	10.7	11.2	13.6
Porphyrio p. pulverulentus (b)	5	23\5	10\1	6\1	1\0		5.0	1.3	1.6	10.7	9.9	12.8	12.3	11.8	13.1	12.6	12.8	11.3	10.5	13.1	11.0	10.7	10.7	11.2	13.6
Porphyrio p. madagascariensis	6	21\4	12\2	12\2	16\3	16\3		3.7	3.4	11.5	10.4	11.5	11.8	11.3	12.6	11.5	12.8	10.8	10.5	9.9	9.9	10.9	10.9	11.2	13.6
Porphyrio p. seistanicus	7	19\4	6\0	2\0	5\1	4\1	12\2		0.3	10.4	9.7	11.5	11.5	11.0	12.3	11.8	12.0	10.0	9.7	11.8	10.2	9.4	9.4	10.4	12.6
Porphyrio mantelli mantelli	8	20\4	5\0	1\0	6\1	5\1	11\2	1\0		10.2	9.9	11.2	11.3	10.7	12.0	11.5	11.8	9.7	9.4	11.5	9.9	9.1	9.1	10.2	12.3
Porzana tabuensis	9	32\7	31\9	29\9	31\10	31\10	35\9	31\9	30\9		6.5	11.0	12.6	12.6	13.4	11.8	12.6	12.3	11.0	11.5	9.2	11.2	11.2	12.0	12.8
Porzana pusilla	10	34\8	29\10	29\10	27\11	27\11	30\10	27\10	28\10	20\5		11.8	12.6	13.1	13.9	12.8	13.1	12.3	12.6	11.8	10.2	11.0	11.5	12.3	14.1
Rallus aquaticus	11	38\3	35\6	36\6	42\7	42\7	38\6	38\6	37\6	33\9	37\8	B	6.8	6.8	6.5	7.6	6.8	7.6	7.1	8.6	9.1	11.5	11.0	12.0	14.1
Rallus phippensis australis/assimilis	12	38\3	36\5	39\5	41\6	41\6	40\5	39\5	38\5	40\8	39\9	23\3		1.1	1.3	2.4	2.1	4.2	5.2	10.2	9.7	9.4	9.4	10.7	12.8
Rallus owstoni	13	38\4	34\5	37\5	39\6	39\6	38\6	37\5	36\5	40\8	41\9	23\3	4\0		1.3	1.3	1.1	4.2	4.7	9.7	10.7	9.7	9.7	11.0	11.8
Rallus sylvestris	14	38\4	38\6	39\6	43\7	43\7	42\6	41\6	40\6	42\9	43\10	21\4	4\1	4\1		2.6	2.4	5.0	6.0	10.0	10.5	10.7	10.2	11.5	12.6
Rallus modestus	15	41\3	37\5	40\5	42\6	42\6	39\5	40\5	39\5	37\8	40\9	26\3	9\0	5\0	9\1		1.8	5.5	5.5	10.0	10.5	10.5	10.5	11.8	12.8
Rallus philippensis dieffenbachii	16	42\3	38\5	41\5	43\6	43\6	40\5	41\5	40\5	40\8	41\9	23\3	8\0	4\0	8\1	7\0		5.0	5.2	9.7	10.7	10.2	10.2	11.5	12.6
Amaurornis inepta	17	37\2	31\4	34\4	38\5	38\5	37\4	34\4	33\4	40\7	39\8	25\4	15\1	15\1	17\2	20\1	18\1		4.7	10.8	11.3	8.9	8.9	11.0	13.1
Gallirallus australis	18	34\3	29\5	32\5	34\6	34\6	35\5	32\5	31\5	24\8	39\9	22\5	18\2	16\2	20\3	19\2	18\2	17\1		9.7	9.4	8.4	8.4	9.2	10.7
Rallina castaneiceps	19	33\4	32\8	35\8	41\9	41\9	30\8	37\8	36\8	33\11	33\12	27\6	32\7	30\7	30\8	31\7	30\7	15\6	30\7		8.9	11.0	10.4	9.9	12.8
Eulabeornis cajanea	20	37\1	35\3	34\3	39\4	38\4	35\3	36\3	35\3	29\6	32\7	32\3	35\2	39\2	37\3	38\2	39\2	42\1	34\2	29\5		10.2	9.6	9.1	13.9
Fulica atra	21	37\6	29\8	28\8	32\9	32\9	34\8	28\8	27\8	32\11	30\12	37\7	32\4	33\4	36\5	36\4	35\4	31\3	28\4	35\7	35\4	C	0.5	6.0	11.5
Fulica c. prisca/chathamenis	22	35\6	29\8	26\8	32\9	32\9	34\8	28\8	27\8	32\11	32\12	35\7	32\4	33\4	34\5	36\4	35\4	31\3	28\4	33\7	33\4	2\0		5.5	11.5
Gallinula chloropus	23	33\9	26\11	27\11	31\12	31\12	32\11	29\11	28\11	32\14	32\15	38\8	34\7	35\7	36\8	38\7	37\7	36\6	28\7	30\8	28\7	20\3	19\3		13.1
Grus antigone	24	36\17	30\17	29\17	34\18	34\18	35\17	31\17	30\17	16\23	30\24	35\19	32\17	28\17	30\18	32\17	31\17	34\16	26\15	32\17	33\20	27\17	27\17	30\20	

or *Porphyrio* as suggested by Olson (1973*a*). *Gallinula chloropus* is always found to be basal with respect to *Fulica* (coot). *Eulabeornis cajanea* arises at the base of *Porzana* and this clade is basally associated with *Porphyrio*. The deep relationships of these taxa are however poorly resolved and not well supported in this analysis.

Neighbour-joining trees were generated from cytochrome-*b* data using either observed distance (figure 3) or with Kimura two-parameter correction and excluding *Gallinula martinica* (see below). Both are topologically similar to the 12S NJ tree (figure 2) except that *Gallirallus australis* and *Amaurornis ineptus* form a couplet within *Rallus*, which is the same result obtained for 12S using PAUP. This couplet was also evident in ML analysis of cytochrome-*b*. A basal split separates a weakly supported *Porzana/Porphyrio* group from *Rallus*, as with 12S data (figure 2). A tree of the same topology was returned from unweighted parsimony analysis, and by both distance and two-colour Hadamard methods. When included in analysis, *Gallinula martinica* is basal to all rails and the position of *Porphyrio* is apparently influenced by this. When NJ analysis is repeated with these taxa using only transversions, *G. martinica* is basal to *Porphyrio* and the position of this clade is consistent with the trees shown. The divergent nature of the cytochrome-*b* sequence from *Gallinula martinica* compared with that of other rails can be seen in table 2, although comparison of translated sequences (not shown) revealed no abnormal amino acid replacements in the sequence from this taxa.

Analysis of 12S sequence data from representatives of rail genera and other avian families indicated an association between *Porphyrio* and *Porzana* and between *Fulica* and *Rallus*. The jacanas (*Jacana jacana* and *Irediparra gallinacea*) were closer to other non-rails, and the kagu (*Rhynochetos jubatus*) branches closest to the sarus crane (*Grus antigone*). The kagu is, as expected, extensively diverged from the crane and lacks any close relatives in this analysis.

SplitsTree networks constructed for the two clades of most interest in this study, *Porphyrio* and south-west Pacific *Rallus*, demonstrate the relationship of flightless and volant species in these groups as indicated from 12S sequences. Amongst Pacific *Rallus* (figure 4), strong support is given to the notion that *R. philippensis dieffenbachii* (from Chatham Island) is a distinct species and it is closer to *R. modestus* (also from Chatham Island) than it is to *R. philippensis*. According to the analyses of the full data set the phylogeny would be rooted, via the weka (New Zealand) and *Amaurornis* (New Guinea) near *R. owstoni* (Guam), indicating that the nearest relatives, in this analysis, of most of the island endemic flightless species are themselves flightless taxa.

The subspecies of *Porphyrio porphyrio* cover a wide geographical area (figure 1), and this is reflected in the relatively larger genetic distances between these taxa (table 3, and figure 5). If *P. p. madagascariensis* (South Africa) is taken as the root of the *Porphyrio* tree (as indicated by 12S/NJ and 12S/PAUP), the flightless *P. mantelli hochstetteri* (South Island New Zealand) is next to branch off, followed by a group that are genetically

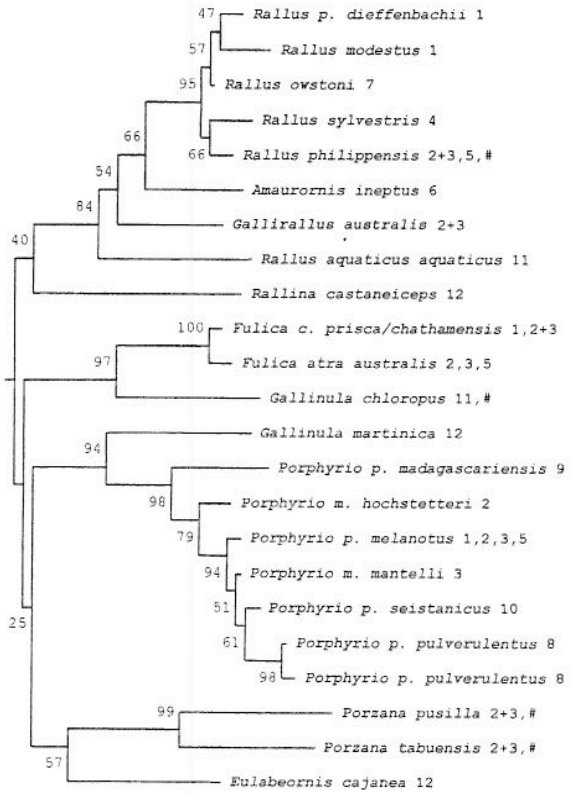

Figure 2. Neighbour-joining tree constructed from observed distances among 12S sequences from several rails and the sarus crane, *Grus antigone* (not shown). Values shown at internal nodes are per cent bootstrap confidence levels (Kumar *et al.* 1993) resulting from 500 replications. Numbers at tips correspond to geographic locations of taxa given in figure 1, and indicate the populations from which samples used in this study originated. The # symbol indicates taxa with wider, unsampled ranges.

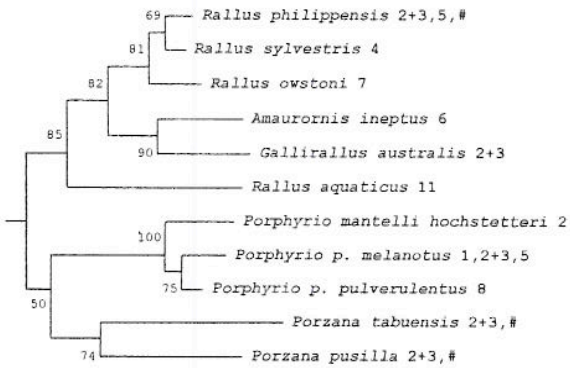

Figure 3. Neighbour-joining tree constructed from observed distances among cytochrome-*b* sequences from several rails and the sarus crane, *Grus antigone* (not shown). Values shown at internal nodes are per cent bootstrap confidence levels (Kumar *et al.* 1993) resulting from 500 replications. Numbers at tips correspond to geographic locations of taxa given in figure 1, and indicate the populations from which samples used in this study originated. The # symbol indicates taxa with wider, unsampled ranges.

similar to one another comprising *P. p. seistanicus* (Turkey), the flightless *P. m. mantelli* (North Island New Zealand) and *P. p. melanotus* (Australia). The volant *P. p. pulverulentus* (Philippines) arises from this group. Given that the volant taxa amongst this latter

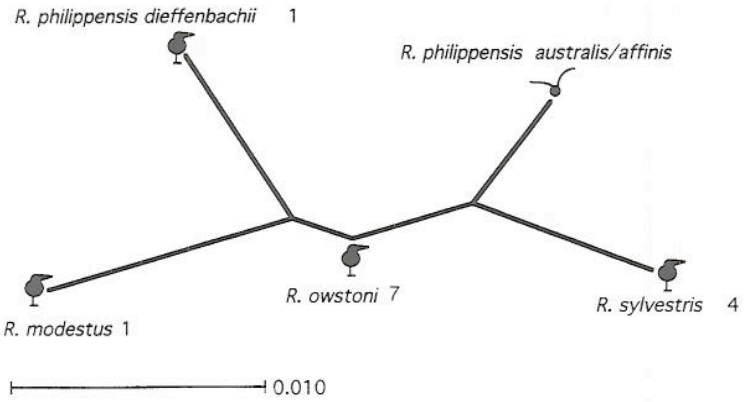

Figure 4. SplitsTree plot of south-west Pacific *Rallus* using observed distance data from 12S sequences. The plot has a 'fit' of 99.9% and included all homologous sites. Numbers at tips correspond to geographic locations given in figure 1, and indicate the populations from which samples used in this study originated. The symbols ✓ and ✗ indicate volant and flightless taxa respectively. Trees of the same topology and scale were returned by ML methods.

group could not have evolved from the flightless North Island (NI) takahe, *P. m. mantelli*, it is apparent that all share a common volant ancestor. In fact, the position of *P. m. mantelli* (NI takahe) on the node from which *P. p. melanotus*, *P. p. seistanicus* and *P. p. pulverulentus* arise indicates that the NI takahe probably carries the 12S genotype of the volant ancestor of those three *P. porphyrio* subspecies. *P. p. melanotus* is genetically very close to this, being separated by a single base substitution unique to this subspecies. It follows therefore that the NI takahe and the South Island (SI) takahe, *P. m. hochstetteri*, are each genetically closer to their respective volant ancestors than to each other.

4. DISCUSSION

(*a*) *Materials and analysis*

Although very many flightless island rails existed until recently, few remain extant. Most of those that do, survive in very low numbers. The development of extraction and PCR techniques has enabled the use of subfossils (Hagelberg & Clegg 1991) and museum skins (Houde & Braun 1988; Taberlet & Bouvet 1991) as sources of DNA, and have thus provided the opportunity for the kind of study presented here. These techniques have recently become valuable and reliable tools in archaeology (Hardy *et al.* 1995; Parr *et al.* 1996) and evolutionary biology (Cooper *et al.* 1996; Taylor 1996). Not all subfossil bones yield DNA of a suitable quantity and quality for PCR and sequencing because they accumulated in conditions that were not suitable for the preservation of DNA. In the present study the quality of subfossil bone specimens varied considerably, as did the ease with which DNA sequence was obtained from them. The 12S primers were more effective for amplifying DNA extracted from bones than were the cytochrome-*b* primers, and sequences could not therefore be obtained for both genes from all extinct species. A number of extinct New Zealand rails are unfortunately absent from the analyses because of the scarcity of suitable quality material at this time, these include *Diaphorapteryx hawkinsii*, *Capellirallus karamu* and *Tribonyx hodgenorum*.

Phylogenetic analysis of the data obtained has produced comparable results with all of the range of up-to-date methods used, and this suggests that reliable interpretations of the data have been achieved. However, deeper nodes in the phylogenetic trees of the taxa examined remain poorly resolved. This situation does not detract from the main objective of this work, although a clearer picture of the relationship of *Gallirallus australis* and *Amaurornis ineptus* would be useful. This could probably be more easily achieved by the inclusion of further congenerics. Most important as far as phylogenetics of the Rallidae is concerned is the implication that clarification of deep relationships will only be acheived through the inclusion of additional nucleotide data (Russo *et al.* 1995).

(*b*) *Phylogenetic reconstruction*

All of the rails used in this study clearly fall together as a group distinct from the crane and the kagu. This accords with evidence from DNA–DNA hybridization (Sibley & Ahlquist 1990) which suggests that the kagu is closer to the cranes than to the rails. Among the rails, the polytomous nature of the phylogeny, although poorly supported, is consistent with the morphological continuum of forms that seem to have few rigorous distinctions between intrafamilial groups (Olson 1973*b*). The topology of the 12S NJ tree is compatible with DNA–DNA hybridization data (Sibley & Ahlquist 1990). Clades within the Rallidae supported by the present data are *Gallinula chloropus* (moorhen) basally with *Fulica* spp. (coots), *Amaurornis* (= *Megacrex*) *ineptus* within *Gallirallus*/*Rallus*, and *Gallinula* (= *Porphyrula*) *martinica* basally with *Porphyrio*. The Northern Hemisphere *Rallus aquaticus* is basal to the south-western Pacific *Rallus*, which show a range of distances between predominantly flightless taxa. Analysis of further members of the northern *Rallus* group will be required to confirm the distinction, if any exists, between *Rallus* and *Gallirallus*. The South American rails, *Rallina castaneiceps* and *Eulabeornis cajanea*, arise from the base of the rails but their affinities are not clear from the present study.

The following three sections deal in detail with clades that in this study include flightless species.

(i) *Coots*

Two giant coots are known from New Zealand, *Fulica chathamensis chathamensis* from the Chatham Islands (Forbes 1892) and *F. c. prisca* from the mainland (Hamilton 1893). The reliability of the depth of the brachial depression of the humerus as a distinguishing osteological character described by Olson (1975) was questioned by Millener (1980), who described alternative characters which he considered sufficient to designate the two forms as species. The present genetic analysis confirms the close relationship of the two flightless coots, as they were found to have identical 12S gene sequences. However, only two nucleotide substitutions separate them from the much smaller, volant coot, *Fulica atra australis*. Although the result is consistent with the proposal that the two flightless forms were derived one from the other (Olson 1975), the two giant coots which existed on islands separated by over 500 km of sea are likely to have been independently derived from the same ancestor. The evolution of flightlessness and gigantism would presumably have precluded subsequent dispersal (Millener 1991).

Fulica atra stock are present in the recent fossil record of Australia (Olson 1975) but not New Zealand and have apparently invaded New Zealand on more than one occasion without establishing permanent populations. Indeed, the *F. atra* DNA sample used in this study came from an individual caught in New Zealand, which was probably a descendant of a recent (circa 1958) colonization event (Small & Soper 1959). Comparison of 12S sequences from the coots and a moorhen (*Gallinula chloropus*) indicates that both of the substitutions which differentiate *F. c. chathamensis/prisca* from *F. atra* were probably derived in the volant *F. atra*. If so, this suggests that morphological evolution of the flightless coots in New Zealand was recent and therefore probably rapid, and presumably occurred in two parallel events. The New Zealand giant coots were not unique and there is evidence that an equivalent pair of giant *Fulica* species evolved on the islands of Mauritius and Reunion in the Indian Ocean (Greenway 1967; Olson 1977).

(ii) *Brown rails*

The western Pacific contains only one volant representative of this group (*Rallus philippensis*) but many flightless species (Diamond 1991), and all have been assigned to the genus *Gallirallus* by Olson (1973*a*). However, Ripley (1977) reserves that genus exclusively for the weka (*G. australis*). No genetic distinction was found between the two described subspecies of weka examined in this study, *G. a. hectori* and *G. a. australis* from Kapiti Island (Beauchamp 1987). Unpublished allozyme electrophoresis data support the view that all weka subspecies are closely related (Rod Hitchmough, personal communication). DNA sequences from two subspecies of *Rallus philippensis* (banded rail) were similarly indistinguishable from one another but clearly distinct from the weka. This indicates that the New Zealand subspecies of the banded rail is a recent arrival. A close relationship of New Zealand and Australian banded rails has previously been proposed by Schodde & de Naurois (1982). Fossil evidence also indicates that the present New Zealand population is the result of very recent colonization (Millener 1981).

The Lord Howe Island woodhen (*R. sylvestris*) and the New Caledonian (*R. lafresnayanus*) (not included in this study) are considered by Ripley (1977, p. 68) to be so alike that they might be relics of a once widely distributed and now all but extinct species. Beauchamp (1989) has a similar view with respect to *R. sylvestris* and *G. australis*. However, Olson (1973*b*, p. 398) believed *R. sylvestris* and *R. lafresnayanus* to have been independently derived, and so diverged as to warrant placing the latter species in a monotypic genus, *Tricholimnas*. It is likely that morphological and behavioural similarities between these species are the result of convergence associated with evolution of a flightless habit (Olson 1973*b*), and this is what the genetic evidence indicates (figure 2). In the present study, the flightless taxa which are most similar to the weka (*R. sylvestris*, *R. owstoni*) were more closely related to the volant banded rail (*R. philippensis*) than to the weka (*G. australis*) which is genetically very distinct.

Genetic analyses including the Northern Hemisphere species *R. aquaticus* show that the *Rallus* clade is paraphyletic with respect to both the *Gallirallus* and *Amaurornis* of Ripley (1977). Olson (1973*a*) places *Amaurornis* (= *Megacrex*) *ineptus* with *Habroptilus wallacei*, whilst Ripley (1977) puts *Habroptilus wallacei* in *Rallus*. Thus, the placing of *Amaurornis ineptus* in the present study within the *Gallirallus/Rallus* 'clade' is consistent with their combined implied conclusions. If *R. sylvestris* and *R. owstoni* are placed within *Gallirallus* along with the weka (*G. australis*) as Olson (1973*a*) suggests, then *Amaurornis inpetus* apparently belongs there too, despite the morphological and behavioural distinctiveness of this flightless rail. However, inconsistency in branching patterns returned by different tree building methods in the present study indicates that the relationship of *Gallirallus australis* and *Amaurornis inpetus* is unresolved.

The flightless Dieffenbach's rail, *Rallus philippensis dieffenbachii*, (Ripley 1977) is, despite morphological similarities (Diamond 1991), quite distinct from the volant *R. philippensis* according to genetic evidence (figure 4). This is consistent with its classification as a distinct species within *Rallus* (Greenway 1967) or *Gallirallus* (Olson 1973*b*). Comparison of osteological material also indicates that Dieffenbach's rail is not a subspecies of *Rallus philippensis* (Trewick, unpublished data). *R. p. dieffenbachii* was found to be genetically most similar to another flightless species, *R. modestus* (figure 4). These two morphologically divergent species were endemic to the Chatham Islands where they existed in sympatry (Millener 1991). It is most likely that these two species evolved from separate colonizations rather than sympatrically, and probable that the ancestor of each was the same volant species. The extent of genetic divergence of the colonizers and the relative timing of their arrival cannot be determined, but the suggestion that the morphologically

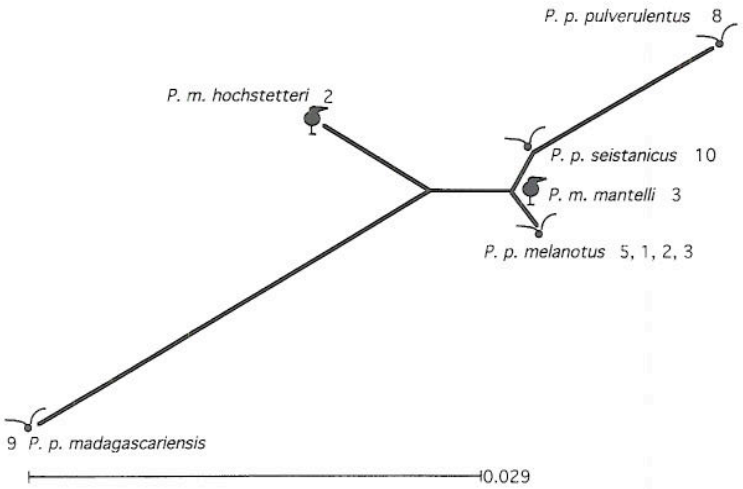

Figure 5. SplitsTree plot of *Porphyrio* using observed distance data from 12S sequences. The plot has a 'fit' of 100% and included all homologous sites. Numbers at tips correspond to geographic locations given in figure 1, and indicate the populations from which samples used in this study originated. The symbols and indicate volant and flightless taxa respectively. Trees of the same topology and scale were returned by ML methods.

'less modified' of the two, *R. p. dieffenbachii*, was the result of a more recent colonization (Olson 1975) by the volant ancestor is not substantiated by the genetic evidence. *R. p. dieffenbachii* and *R. modestus* are genetically more divergent from one another and other taxa than some other flightless species are from volant taxa (e.g. *R. philippensis* versus *R. owstoni*).

(iii) *Purple swamphens*

The American purple gallinule was put in the genus *Gallinula* by Ripley (1977) but has otherwise been placed close to other purple rails in *Porphyrula* or *Porphyrio* (Mayr 1949; Olson 1973*b*). The present analysis demonstrates that the appellation (purple) 'gallinule' is misleading, and the species is indeed more appropriately placed in *Porphyrio*, or retained in *Porphyrula* because it is genetically very distinct from the moorhen, *Gallinula chloropus* (figure 2). According to Ripley (1977) the genus *Porphyrio* comprises two species; the polytypic *Porphyrio porphyrio* consisting of 13 volant subspecies, and *Porphyrio mantelli* which consists of two flightless subspecies restricted to New Zealand. Although takahe were widespread until recent times (Beauchamp & Worthy 1988), *Porphyrio m. hochstetteri* is now restricted to southern mountains of the South Island of New Zealand. A second takahe morphotype is known exclusively from subfossil bones collected in the North Island of New Zealand. Osteometric analysis indicates that it is distinct from *P. m. hochstetteri*, and it has been proposed that these subspecies should be considered as separate species independently derived from a volant *Porphyrio* ancestor (Trewick 1996).

In New Zealand, *Porphyrio porphyrio* is currently represented by the Australian subspecies *P. p. melanotus*, which is thought to have colonized New Zealand approximately 300 years ago (Millener 1981). *Porphyrio porphyrio* sampled from Chatham Island was genetically indistinguishable from *P. p. melanotus* sampled from Australia and New Zealand and this is consistent with recent dispersal between these areas. It is interesting to note that *P. p. melanotus* does not share the apparently ancestral trait of bronze/green dorsal plumage that is exhibited by several of its sister subspecies (including the African *P. p. madagascariensis*), *Porphyrula* (notably '*Gallinula*' *martinica*) and its geographically closest relative, the extant takahe *P. m. hochstetteri*. Molecular phylogenetic analyses using both cytochrome-*b* and 12S sequences place *P. m. hochstetteri* (South Island New Zealand) as an earlier derivation of the *Porphyrio* clade than *P. p. melanotus*. In this analysis *P. p. madagascariensis* of central and southern Africa appears to be the oldest and *P. p. pulverulentus* (Philippines) the most derived. A single dispersal event out of Africa is consistent with this, but the direction and sequence of subsequent dispersal events cannot be determined from the present data. *P. p. seistanicus* (Turkey), *P. m. mantelli* (North Island New Zealand) and *P. p. melanotus* (Australia and New Zealand) are closer to one another on the tree in figure 5 than to the other *Porphyrio*. A single, apparently derived nucleotide substitution on the *P. p. melanotus* lineage distinguishes this species from the extinct North Island takahe, *P. m. mantelli*. *Porphyrio porphyrio* is paraphyletic with respect to the two takahe if *Porphyrio porphyrio* is retained as a single species. This perhaps surprising result is consistent with morphological evidence that the two takahe species evolved independently from similar volant ancestors, and that *P. m. hochstetteri* was derived from an earlier colonization event (Trewick 1996). The genetic distinctiveness of *P. m. hochstetteri* with respect to *P. p. melanotus* is mirrored by that of their respective feather lice (Ricardo Palma, personal communication; see also Balouet 1991).

If one accepts the above findings then the two takahe should be returned to specific status as *P. mantelli* (extinct) and *P. hochstetteri*. The subspecies of *Porphyrio porphyrio* should also be redefined as species, otherwise *Porphyrio porphyrio* will remain paraphyletic. They appear to possess sufficient distinguishing genetic and morphological markers (Cracraft 1983; McItrick & Zink 1988) to indicate that each is following a

distinct evolutionary trajectory (Wiley 1978). Furthermore, they are likely to remain physically isolated long enough to become (if they have not done so already) morphologically and/or behaviourally (if not genetically) incapable of interbreeding (Mayr 1969). This is the case with *Porphyrio m. hochstetteri* and *Porphyrio p. melanotus* which, although they are now locally sympatric, do not voluntarily interact or attempt to breed.

The 12S sequences from two specimens of *P. p. pulverulentus* showed polymorphism at two sites, one a single base insertion. By comparison, two specimens of *P. p. melanotus* from New Zealand, one from the Chatham Islands (given as a distinct subspecies *P. p. chathamensis* in Ripley (1977)) and one from Australia showed no nucleotide differences. Finding polymorphism within *P. p. pulverulentus* demonstrates that not all populations of *Porphyrio* are equally homogenous, that there may be greater genetic differentiation within taxa than is indicated by this study, and that the 12S molecule can be prone to unexpectedly high levels of variation amongst closely related taxa (Simon *et al.* 1997).

(*c*) *Biogeographical implications*

There is little evidence to support a vicariant origin for any of the flightless rails in the present study. All have volant relatives which tend to be behaviourally flightless, and this provides one explanation for the apparent frequency with which flightless rails evolve. Similarity in form and behaviour of flightless rails is the expected result of convergence or parallelism. Only in weka is there any evidence of radiation within a flightless taxon, and in this case variation in plumage coloration is allopatric and not accompanied by significant genetic variation. This indicates that the weka subspecies are of very recent origin, perhaps within the time frame of the current postglacial period which began approximately 14000 years ago. This is despite an ancient derivation of the weka lineage itself. Allopatric taxa that are in gross morphological terms similar to one another, such as *G. australis*, *R. sylvestris* and even *R. p. dieffenbachii*, are apparently more closely related to their respective volant ancestors than to one another, and have originated over a broad timescale. Even the two forms of takahe, which appear so similar, and which at one time existed in such close proximity to one another, are apparently not directly related to one another. This coincidence of form within this taxon is further supported by subfossil evidence of other takahe-like birds around the world. Flightless '*Porphyrio*' are known from New Caledonia (Balouet 1984; Balouet 1991; Balouet & Olson 1989), Lord Howe Island (White 1962; Greenway 1967; Fuller 1987) and possibly also the island of Reunion (Olson 1977; Fuller 1987). This form of parallelism has also been proposed as one possible explanation for the origin on separate islands of two forms of the most famous of all flightless birds, the dodo (Livezey 1993).

Where related flightless taxa have existed in sympatry (e.g. New Zealand and the Chatham Islands), it is evident that selection, perhaps through competition, has tended to lead to morphological divergence such that species became specialized and distinct despite relatively close genetic affinity. A particularly good example of this is the two small, sympatric Chatham rails (Trewick, in preparation). *Amaurornis ineptus* appears to contradict the generality of conservatism in allopatry in the present data set, being relatively close to the weka genetically yet morphologically distinct. However, unlike other flightless rails, *A. ineptus* occupies an island where predators are present and appears to have become specially adapted to clambering amongst mangroves (Ripley 1977).

Taxa derived through a process of peripheral allopatric speciation are expected to display a combination of shared ancestral (symplesiomorphic) and uniquely derived (autapomorphic) characters (Brooks & McLennan 1991). This is true for the flightless rails as a group but it is evident that many genetically related flightless species share uniquely or independently derived characters, and have therefore evolved through a process of parallelism rather than convergence. Island evolution of flightlessness can be considered an extreme form of allopatric speciation because flightlessness fixes the species in space and prevents gene flow. Dispersal and colonization may occur frequently, but not so frequently as to maintain gene flow preventing speciation. The frequency of founding dispersal is clearly high, as indicated by the almost universal occupation of habitable islands by land birds. The evidence given above indicates repeated dispersal events to some islands, with the conclusion that speciation through loss of flight is very rapid (Olson 1973*a*; Feduccia 1980). The process by which this takes place remains unclear (Trewick 1995).

In the rails genetic distances between volant and flightless species were found to be as small as 0.3% (between 12S sequences of *P. m. mantelli* and *P. p. melanotus*, table 3). This is suggestive of the rapidity with which evolution of radically distinct and gigantisized flightless forms may occur (Olson 1973*a*). The estimation of temporal divergence from such small values is unreliable, and the 12S gene may be particularly poor as a molecular clock for closely related taxa (Simon *et al.* 1996). Estimates based on rates of 12S evolution determined from other avian taxa (Cooper 1994) indicate a divergence time for *P. m. mantelli* and *P. p. melanotus* around one million years ago. But, given the small genetic distances involved the estimate is likely to be approximate and the actual divergence time may well be far less. Sibley & Ahlquist (1990) estimated the divergence of *P. m. hochstetteri* and *P. porphyrio* at less than two million years from DNA–DNA hybridization.

Flightless rails are not necessarily all recently evolved. In genetic terms the weka is well separated from related and morphologically similar flightless rails such as the Lord Howe woodhen (*R. sylvestris*). Olson (1973*a*) argued that morphological examination fails to determine the order of their derivation because the plumage of one is more derived whilst the skeleton of the other is apparently most derived. McGowan (1986) states: 'We could not determine the point in time when

wekas became flightless because there is nothing in the skeleton today to suggest that they are flightless'. Comparing bones of two Chatham rails (*R. p. dieffenbachii* and *R. modestus*) Olson (1975) concluded that *G. dieffenbachii* was 'obviously the more recent arrival'. The genetic evidence presented here does not indicate that either is substantially older than the other, and the degree of morphological specialization of a species should not necessarily be taken as an indication of its time of origin. Flightless rails are specialized to an extent dictated by the nature of the selective environment and competitors.

Evolution of flightlessness is both rapid and frequent in the right circumstances, and it is a process which is repeated as and when suitable colonizers reach islands. Morphological change associated with evolution of flightlessness is an adaptive response to selection pressure. It also has the effect of rapidly isolating island endemics from genetic introgression with closely related volant species, despite potential ecological overlap, as is apparent from the incidence of repeated colonization events. The nature and repeatability of this process endorses Darwin's prime concept of descent with modification, but the rapidity with which speciation can occur in such circumstances and that molecular techniques have helped to reveal, goes far beyond what he was able to predict.

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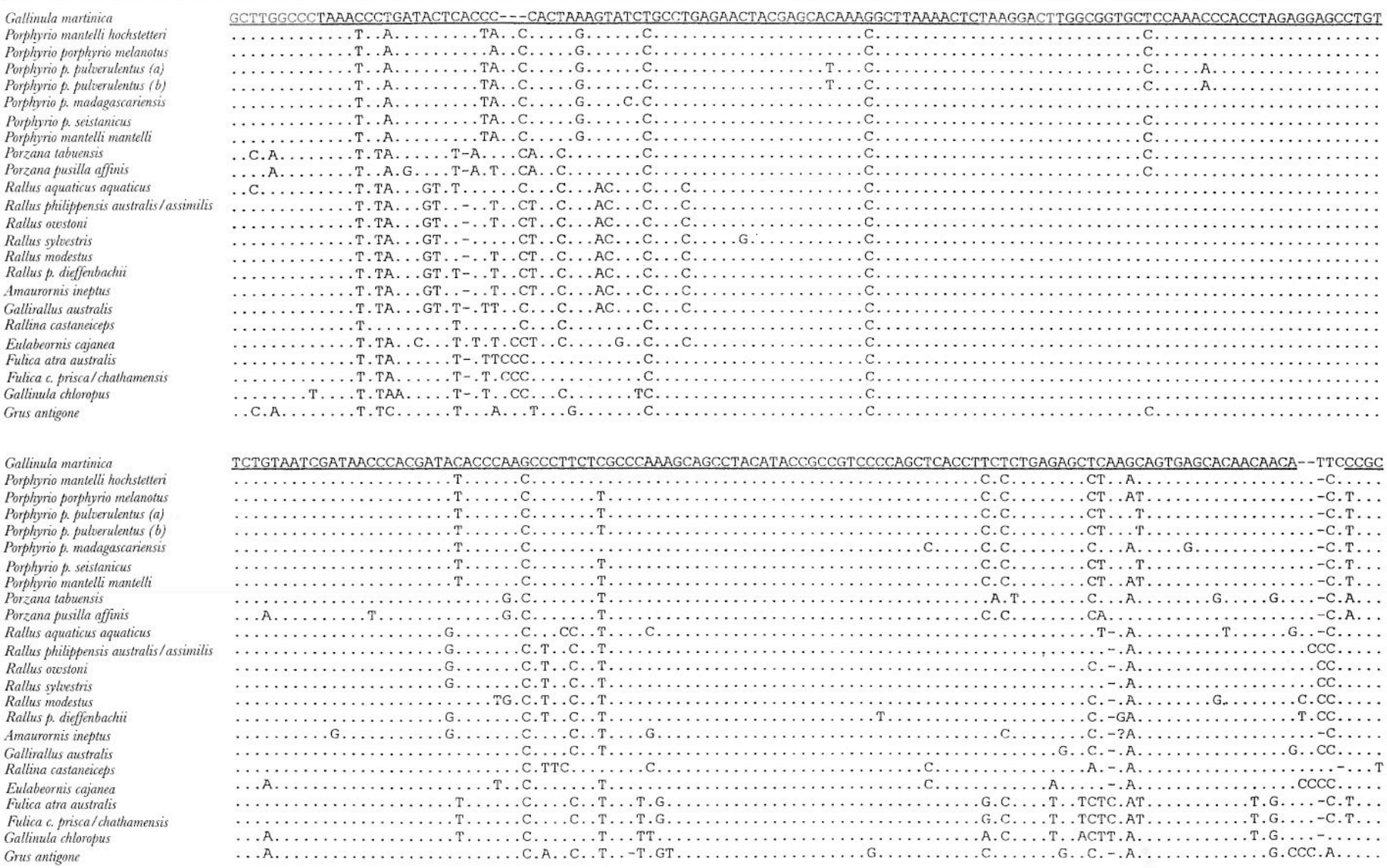
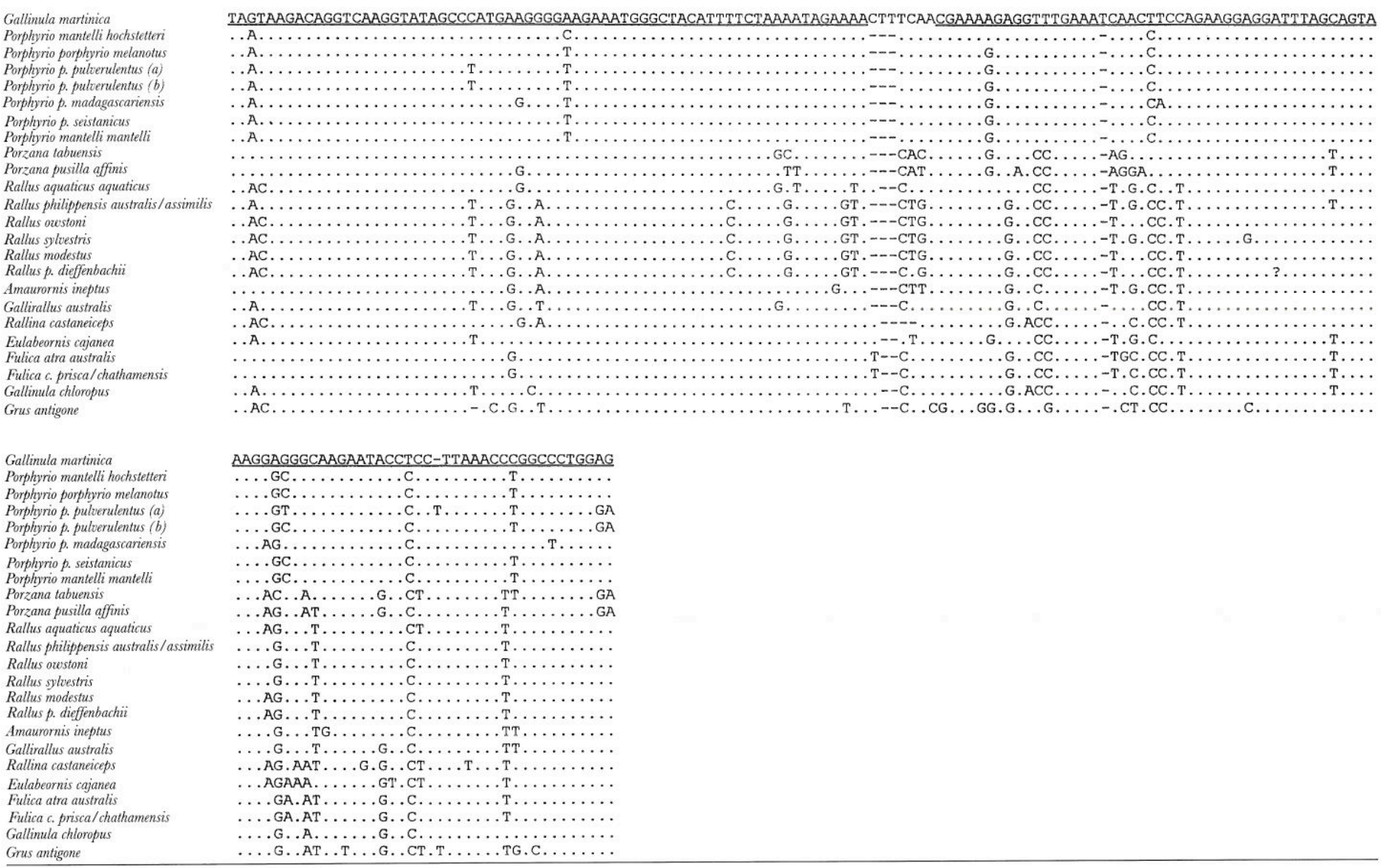
APPENDICES (see pp. 444–446)

Appendix (*a*) Aligned 12S sequences of 23 rail taxa and sarus crane (*Grus antigone*) used in this study corresponding to positions 1753–2146 in the chicken mtDNA sequence (Desjardins & Morais 1990). The positions underlined in the *Gallinula martinica* sequence were identified as homologous in all taxa and were used for subsequent analysis. Alignments were made with comparison to secondary structure models (see text). Dots indicate sites have the same nucleotide as the reference sequence (*Gallinula martinica*).

Appendix (*b*) Aligned 12S sequences of sarus crane, two species of jacana, spurwing plover, and kagu, respectively. The positions underlined in the *Grus antigone* sequence were identified as homologous in these taxa and representatives of the Rallidae, and were used for subsequent analyses.

Appendix *(a). MtDNA 12S sequences of rails and sarus crane (Grus antigone)*




Gallinula martinica
Porphyrio mantelli hochstetteri
Porphyrio porphyrio melanotus
Porphyrio p. pulverulentus (a)
Porphyrio p. pulverulentus (b)
Porphyrio p. madagascariensis
Porphyrio p. seistanicus
Porphyrio mantelli mantelli
Porzana tabuensis
Porzana pusilla affinis
Rallus aquaticus aquaticus
Rallus philippensis australis/assimilis
Rallus owstoni
Rallus sylvestris
Rallus modestus
Rallus p. dieffenbachii
Amaurornis ineptus
Gallirallus australis
Rallina castaneiceps
Eulabeornis cajanea
Fulica atra australis
Fulica c. prisca/chathamensis
Gallinula chloropus
Grus antigone
TAGTAAGACAGGTCAAGGTATAGCCCATGAAGGGGAAGAAATGGGCTACATTTTCTAAAATAGAAAACTTTCAACGAAAAGAGGTTTGAAATCAACTTCCAGAAGGAGGATTTAGCAGTA
AAGGAGGGCAAGAATACCTCC-TTAAACCCGGCCCTGGAG

Appendix *(b). MtDNA 12S sequences of non-rails*

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Grus antigone                 GCCTAGCCCTAAATCTCGATACTTACC-ATACTGAAGTATCCGCCTGAGAACTACGAGCACAAACGCTTAAAACTCTAAGGACTTGGCGGTGCCCCAAACCCACCTAGAGGAGCCTGTTCTATAATCGAT
Irediparra gallinacea          ..T.G..........A...GT..-.TTCC..CA..AC.......C.............G.....................................T.....................G........
Jacana jacana                 ..T.G...T.......A...GT..-.TCC...CA..AC.........G.....................................!.....................................
Vanellus miles novaehollandiae ..T............C....G.......C...CA..CG.......C.....................................................................
Rhynochetos jubatus           ................A..CG..A..T.C....CT..CG......C.............T.....................................................C..........

Grus antigone                 AACCCACGATACACCCAACCACTCCTTGCT-AGTGCAGCCTACATACCGCCGTCGCCAGCTCACCTCCTCTGAGGGCCCAACAGTGAGCACAACAGCC--CCT-ACCCGCTAACAAGACAGGTCAAGGTA
Irediparra gallinacea          ..T.....T.......G...T...........AAA.....................T....C.....TTA....AA.AA...T....G..TT.........-.C.........G...........G.....
Jacana jacana                 ........T.......GG..G........CA.ACA.......T.....................TG.....A.TATT.G.......TT..T.......TC.....................G.....
Vanellus miles novaehollandiae ................G...........C..AG.....................................T...AA..AA.....................TA.TCA........G.................
Rhynochetos jubatus           ..........T........C.C...A..A...GT.....................C....CT.A.......GAT..T....GA..A..T-..T...T..---......T.................

Grus antigone                 TAGCC-ACGGAGTGGAAGAAATGGGCTACATTTTCTAAAA-TAGATAACCC-ACGGAAGGGGGGTGTGAAACCTCCCCCAGAAGGCGGATTTAGCAGTAAAGGGGGATAAT-AATGCCCTCTTTAAACTG
Irediparra gallinacea          .....C.T....A.....T.........................AT..TTT......A....C.........A....TG.................T......T..A.C...GT.A.T..A......GT..
Jacana jacana                 .....T......A.................................AC..T.....-..A............T.A...?T.....................C.A..C...........G......GTC.
Vanellus miles novaehollandiae .....C.T.............................C......AG.G.-....-G.A...A..........A.T.........................C.AA-C......A...TG......GT..
Rhynochetos jubatus           .....T.T..G.C......G.................GC.G....AC.TT-....-G..C............A.T..T.A....T........T.....GA............A....CTC.G..GAC.

Grus antigone                 GCCCTGGAG
Irediparra gallinacea          .......G.
Jacana jacana                 ..T....G.
Vanellus miles novaehollandiae ......AG.
Rhynochetos jubatus           ..T....G.
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