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Independent origins of New Zealand moas and kiwis

(ancient DNA/mitochondrial DNA/ratites)

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ABSTRACT **Two groups of flightless ratite birds existed in New Zealand during the Pleistocene: the kiwis and the moas. The latter are now extinct but formerly included 11 species. We have enzymatically amplified and sequenced approximately 400 base pairs of the mitochondrial 12S rRNA gene from bones and soft tissue remains of four species of moas as well as eight other species of ratite birds and a tinamou. Contrary to expectation, the phylogenetic analysis shows that the kiwis are more closely related to Australian and African ratites than to the moas. Thus, New Zealand probably was colonized twice by ancestors of ratite birds.**

The ratites are large flightless birds with a flat breastbone and an archaic palate. Fossil evidence is consistent with the possibility that the ancestors of ratites were flying birds living about 60–50 million years ago and having a keeled breastbone as well as an archaic palate (1, 2). The 10 living species of ratites are the ostrich (in Africa and formerly Asia), the emu and three cassowaries (in Australia and New Guinea), two rheas (in South America), and three kiwis (in New Zealand). According to indirect biochemical methods, these species are related to one another and to tinamous, which are the only contemporary flying birds with an archaic palate (3–5). This article presents direct evidence of genetic relatedness not only for these birds but, more important, for the moas.‖ These are an extinct group of 11 ratite species that lived during the Pleistocene era in New Zealand and on the basis of biogeographic and morphological reasons are generally assumed to share a common ancestor with the kiwis (6, 7).

MATERIALS AND METHODS

Tissue Samples. Moa samples were as follows (see ref. 6): for *Anomalopteryx didiformis* (moa 1) skin and muscle, Southland Museum, Invercargill, New Zealand; for *Pachyornis elephantopus* (moa 2) muscle from specimen 381A, Cambridge University Zoological Museum, Cambridge, U.K.; for *Dinornis novaezealandiae* (moa 3), the Tiger Hill specimen of Yorkshire Museum, York, U.K.; for *Megalapteryx didinus* (moa 4) skin fragments, a rib bone, and muscle tissue from NZM S23808 found on Mt. Owen, National Museum, Wellington, New Zealand (8); skin and tendon from NZM S400, found near Cromwell; tendon from C.68.2., New Zealand; for *Emeus crassus* (moa 5) varnished skin and tendon, Otago Museum, Dunedin, New Zealand. Frozen tissue samples of ostrich (*Struthio camelus*), cassowary (*Casuarius casuarius*), rhea 1 and 2 (*Rhea americana* and *Pterocnemia pennata*, respectively), and tinamou (*Eudromia elegans*) were from the collection of A.C.W. A blood sample of emu (*Dromaius novaehollandiae*) was provided by P. Baverstock, South Australia Museum. Erythrocytes of kiwis 1–3 (*Apteryx haastii*, *Apteryx owenii*, and *Apteryx australis*, respectively) were from the New Zealand National Frozen Tissue Collection (National Museum, Wellington, New Zealand).

DNA Extraction. Tissue samples were extracted as described (9). The erythrocytes were diluted 1:1 with H_2O and digested in 10 mM Tris·HCl, pH 8.0/1 mM ethylenediaminetetraacetate (EDTA)/100 mM NaCl/0.1 mg of proteinase K per ml/1% sodium dodecyl sulfate. Two independent extractions were carried out from each museum sample. From 2 g of bone of moa 4 soft tissues were removed by using sandpaper and a scalpel blade. The sample was then ground in an acid-washed (1 M HCl for 15 min, two washes with H_2O for 15 min each) mortar and pestle under liquid nitrogen. The powdered bone was dissolved in 40 ml of 0.5 M EDTA, pH 8.0, for approximately 30 hr. The solution was then placed in boiled Spectra/por 3 dialysis tubing (Spectrum Medical Industries) and dialyzed against 4 liters of 10 mM Tris·HCl, pH 8.0 for 24 hr with three changes of buffer. The sample was concentrated by placing it in 400 ml of 20% (vol/vol) polyethylene glycol/10 mM Tris·HCl, pH 8.0 for 6 hr. It was retrieved from the dialysis tubing with 6 ml of extraction buffer and digested and extracted as the soft tissues were.

PCR and Sequencing. Primers used for enzymatic amplification were as follows:

12*S*Bm	(H2148)	5′-GAGGGTGACGGGCGGTGTGTGCAT-3′
12*S*E	(L1873)	5′-ACCCACCTAGAGGAGCCTGTTC-3′
12*S*F	(H2023)	5′-AGAAAATGTAGCCCATTTCT-3′
12*S*G	(H1892)	5′-GGCAAGAGATGGTCGGGTGTA-3′
12*S*H	(H1985)	5′-CCTTGACCTGTCTTGTTAGC-3′
12*S*I	(L1944)	5′-TACATACCGCCGTCGCCAGCCC-3′
12*S*J	(L1999)	5′-CCCCCGCTAACAAGACAGGT-3′

Primers L1091, H1478, L1373 have been previously described (10, 11). The letters L and H refer to the light and heavy strands, respectively, and the numbers within parentheses refer to the position of the 3′ base of the primer in the complete chicken mitochondrial DNA sequence (12). Numbers for the previously published primers refer to the complete human mitochondrial DNA sequence (13). The PCR was performed and products were sequenced from both sides

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¶Deceased July 21, 1991.

‖The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X67626–X67638).

as in ref. 11. Sequences were aligned by eye, using the computer program ESEE (14).

Sequence Analysis. The maximum-likelihood analysis was performed by using the DNAML program (15). To obtain the best fit to the data, transition-to-transversion ratios varying from 1.0 to 30.0 were tested. The branching structure remained the same irrespective of this ratio. However, the estimated likelihood was maximal for a transition-to-transversion ratio of approximately 7.0. Since there is no significant difference in the likelihood estimates for transition-to-transversion ratios between 4.0 and 15.0, it is at present not possible to obtain a good estimate for the transition/transversion bias. When a maximum-parsimony analysis was performed by using PAUP (16) or DNApenny of the PHYLIP package, one most parsimonious tree of 130 changes with the same topology as the maximum likelihood tree was found. Parsimony trees constructed from 1000 bootstrap replications also corroborate the topology of the tree. The moa group occurs in 95% of all replicates, the kiwi group in 96%, the kiwi, emu, and cassowary group in 82%, and the latter group together with the ostrich in 95% of all bootstrap trees. A grouping of kiwis and moas was not observed in any of the bootstrap trees.

RESULTS

DNA Extraction and Sequencing. We have extracted DNA from bones and soft tissue remains of five species of moas representing different genera: *Anomalopteryx*, *Pachyornis*, *Dinornis*, *Megalapteryx*, and *Emeus* (below termed moas 1–5, respectively). In the case of moa 4, soft tissues were obtained from three different animals. A bone sample was also removed from one of the carcasses, which has been carbon-14 dated at 3350 ± 70 years B.P. (8). Nucleic acids were extracted from soft tissue remains of all the birds as described (9) and from the bone sample by a procedure using EDTA (17). Several sets of primers were used for enzymatic amplification of DNA sequences of various lengths from the mitochondrial 12S rRNA gene. It was found that the DNA extracts from modern samples allowed amplifications of longer pieces of DNA than ancient DNA samples (Fig. 1). This is in agreement with previous experience and is most likely caused by chemical damage present in old DNA (18). However, the bone extract allowed amplification of DNA fragments that were 438 bp long (Fig. 1). This was in stark contrast to the extracts of soft tissues (either from the same animal or from other animals), which permitted only poor amplification of DNA fragments that were over 150 bp in length. Thus, it seems that DNA in specimens preserved under similar conditions may survive better in bone than in soft tissues. DNA sequences determined from bone, from soft tissues of the same specimen, and from another individual of the same species were identical but different from other ratite sequences. Preliminary work indicates that moa bones without soft tissue remains may also yield amplifiable DNA. These findings lend support to recent reports that comparatively long mitochondrial DNA sequences can be amplified from old bones of humans (17, 19, 20).

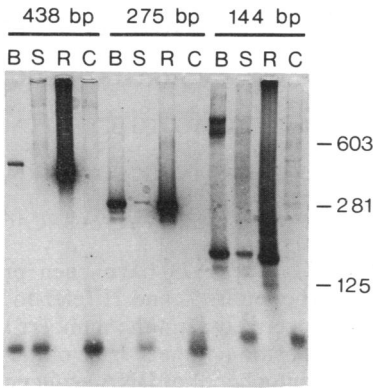

FIG. 1. Agarose gel electrophoresis of amplification products obtained from bone (B) and skin (S) extracts of a 3300-year-old moa as well as from a contemporary rhea (R) and a control extract (C). The molecular sizes of the amplification products are given above the lanes (bp, base pairs). Primers used were L1091–12SBm (438 bp), L1091–H1985 (275 bp), and L1373–12SBm (144 bp). The migration positions of molecular size markers are given in bp.

Ten primers were used to amplify and directly sequence subsections of an approximately 390-bp segment of the mitochondrial 12S rRNA gene from the five moa species. For moa 5, three extracts consistently gave very weak amplifications, many of which proved difficult to sequence. For the remaining four species, extracts yielded amplification products that could be easily visualized and sequenced. In the latter cases, a minimum of one additional extract was prepared from each specimen and found to produce sequences identical to the first extract. In addition, the corresponding DNA sequence was determined for the three kiwi species, *Apteryx haastii*, *A. owenii*, and *A. australis* (termed kiwi 1–3, respectively) as well as the ostrich, the emu, one species of cassowary (*Casuarius casuarius*), and the two species of rhea, *Rhea americanus* and *Pterocnemia pennata* (termed rhea 1 and 2, respectively). As an outgroup, a tinamou was used. The base compositions of all the taxa proved to be statistically indistinguishable from each other. The aligned sequences are presented in Fig. 2.

Phylogenetic Analysis. The distance matrix (Table 1) shows that the four moa species, the three kiwi species, and the two rhea species stand out as groups in which the members are separated by 2–15 transitions and 0–1 transversions. All other pairwise comparisons show higher numbers of evolutionary changes. This result implies that moas and kiwis each represent monophyletic groups. As the number of transversions rises for the comparisons of different species, a progressive fall in the relative amount of transitions is observed due to multiple substitutions at the same sites (22). This confirms that the transition bias which has been shown to exist in mitochondrial DNA of many groups of animals (10) pertains also to ratite birds.

Fig. 3 presents the most likely unrooted phylogenetic tree that can be constructed for the 12S rRNA sequences. When the tinamou is added to the tree it falls on the lineage leading to the rheas. When in addition, the chicken sequence is added to the analysis, it connects to the tinamou line. This reinforces the view that has emerged from indirect biochemical comparisons (3–5) and cytogenetics (23) that ratites represent a monophyletic group, whose nearest relatives are tinamous. Furthermore, it demonstrates that moas fall within the ratite group, as was recently indicated also by the determination of 11 N-terminal amino acids of osteocalcin extracted from bones of moa 4 (24). The tree also confirms that kiwis as well as moas are monophyletic and lends support to the idea that there exists an "Australasian group" of ratite birds (4) that contains the emu and cassowary as well as the kiwis. Closely related to these is the ostrich, while the South American rheas as well as the moas fall outside this group. This suggests that the rheas, the moas, and the ancestor of the Australasian group (including the ostriches) diverged from one another early in ratite evolution.

DISCUSSION

The most surprising result of the phylogenetic analysis is that the two groups of New Zealand ratites have different origins, the moas representing an earlier divergence among ratites, whereas the kiwis more recently shared an ancestor with the

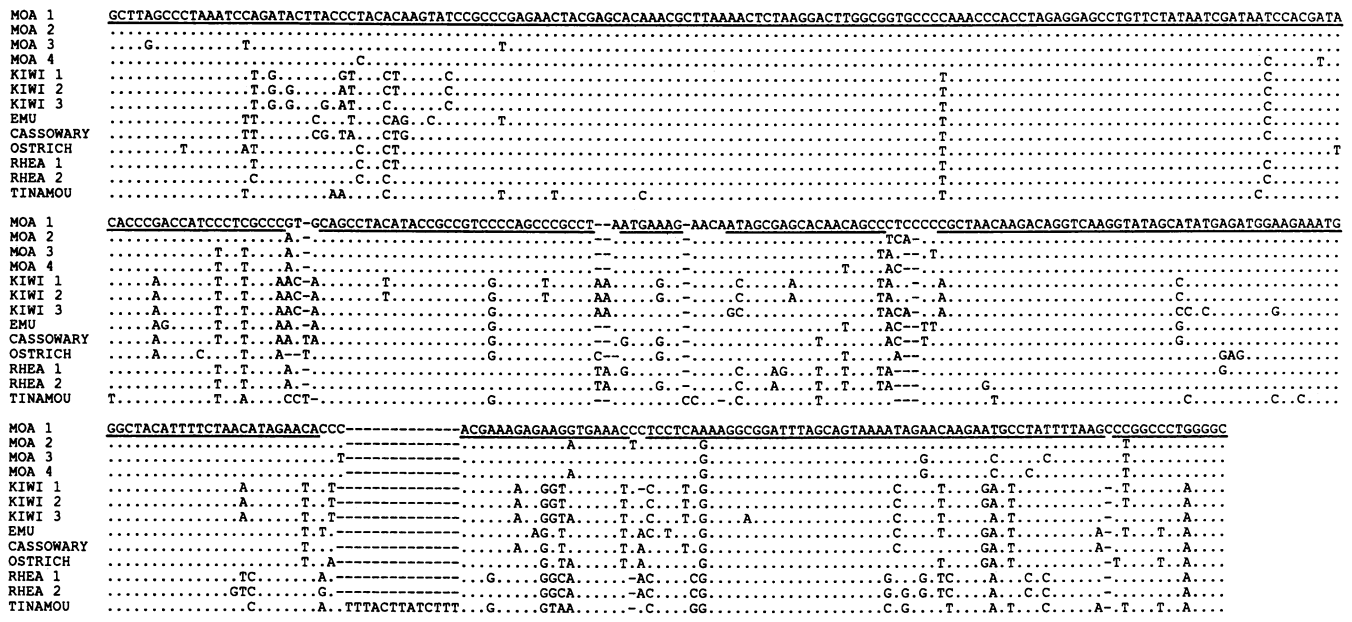

FIG. 2. Partial sequences of the mitochondrial 12S rRNA gene for 12 ratite species and the tinamou, corresponding to positions 1753–2147 in the published chicken sequence (12). Dots indicate identities with the moa 1 sequence. Underlined positions were used for phylogenetic analyses.

ostrich and Australian ratites. This conclusion is supported by the branch lengths of the maximum-likelihood tree, where the internal branch separating the ostrich and Australasian lineages from the rest of the species as well as the internal branch separating the two latter from each other are both of significantly positive length. This is further borne out by a bootstrap test (25) using parsimony, which supports the monophyly of the Australasian ratites and the ostrich at the 95% level.

It is interesting to compare this view of ratite evolution with the view that emerges from morphology. A cladistic analysis of 83 postcranial skeletal characters (26) agrees with the molecular data in that kiwis, emus, and cassowaries form a monophyletic group and that moas constitute an early divergence among ratites. However, most other workers have reached the conclusion that kiwis are associated with moas rather than with the Australasian ratites (27). Since the majority of the morphological traits shared by moas and kiwis are primitive (26), we suggest that kiwis and moas may have evolved convergently from ancestors that were not highly specialized. This may have happened as an adaptation to a noncursorial way of life made possible by the forested habitat and the absence of ground-dwelling predators in New Zealand. Furthermore, whereas the amounts of sequence divergence that are observed among the moa species (3–11 transitions, 0–1 transversions) and among the kiwi species (2–15 transitions, 0–1 transversions) are very similar, the moas show a much larger morphological divergence than the kiwis. This has caused the moas to be classified into different genera (7), while the three kiwis are congeneric. It is tempting to speculate that the discrepancy in morphological diversity between kiwis and moas was caused by the more varied range of niches available to the predominantly herbivorous moas compared with the nocturnal insectivorous kiwis.

It has been claimed that the ancestor of modern ratites was flightless (27) and that the major ratite groups evolved due to the break-up of Gondwanaland. However, the fact that moas and kiwis do not constitute a monophyletic group is incompatible with the view that all branching events between major groups of ratites were caused by continental break-ups. Under the assumption of a flightless ratite ancestor, the moas, which represent an early divergence among the ratites, and the kiwis, which represent a later divergence, must have been isolated together when New Zealand was separated

Table 1. Pairwise comparisons of transitions and transversions in ratite mitochondrial DNAs

	M1	M2	M3	M4	K1	K2	K3	E	C	O	R1	R2	T
Moa 1 (M1)	—	0	0	1	10	10	11	7	7	8	7	7	14
Moa 2 (M2)	3	—	0	1	10	10	11	7	7	8	7	7	14
Moa 3 (M3)	11	10	—	1	10	10	11	7	7	8	7	7	14
Moa 4 (M4)	11	8	10	—	11	11	12	8	8	9	8	8	15
Kiwi 1 (K1)	29	28	29	29	—	0	1	5	5	6	9	9	16
Kiwi 2 (K2)	30	29	30	30	2	—	1	5	5	6	9	9	16
Kiwi 3 (K3)	29	28	31	29	15	13	—	6	6	7	10	10	17
Emu (E)	29	28	27	27	22	23	27	—	2	3	6	6	15
Cassowary (C)	26	27	28	28	18	19	22	17	—	3	6	6	15
Ostrich (O)	23	22	28	23	26	27	30	24	24	—	7	7	16
Rhea 1 (R1)	27	26	25	19	30	31	30	36	34	29	—	0	13
Rhea 2 (R2)	26	25	24	18	32	31	30	37	36	31	10	—	13
Tinamou (T)	24	21	21	25	33	33	29	27	29	30	25	24	—

The numbers of transversion differences are given above the diagonal and the numbers of transition differences are given below the diagonal for the aligned parts of the mitochondrial 12 S rRNA gene. Differences were calculated by using the computer programs PAIRCOMP and COMPSTAT (21).

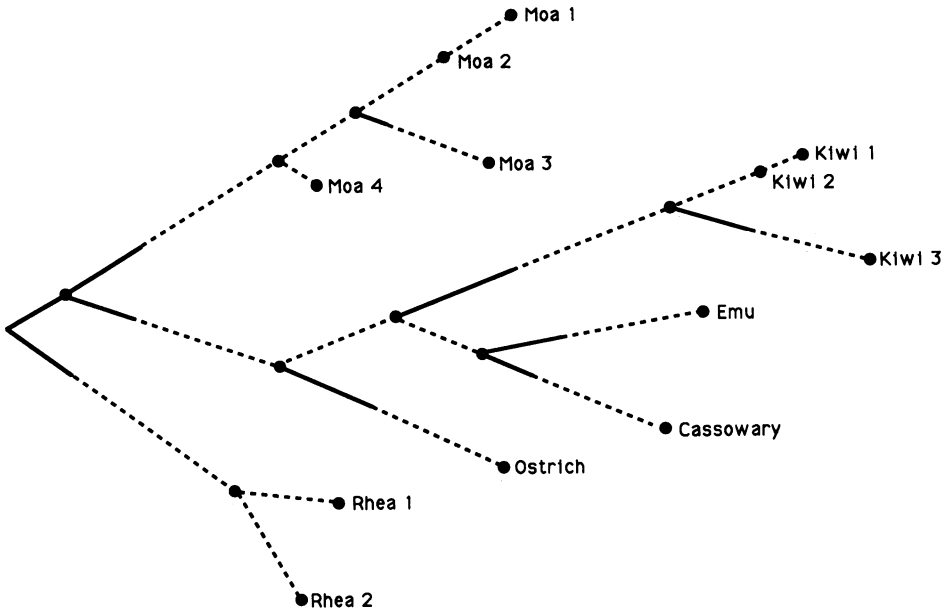

FIG. 3. Unrooted maximum-likelihood tree for 12 ratite birds. The branch lengths are proportional to the expected number of substitutions. Broken and solid lines together represent the estimated length. All shown branch lengths are significantly positive ($P < 0.01$). Solid lines in the tree represent the lower bound of the approximate confidence limit of the branch. When the tinamou sequence is added to the ratite tree, it connects to the rhea lineage at the point indicated by the bend on the branch leading to the rheas.

from Australia at approximately 80 million years ago (28, 29). A more unlikely alternative would be that a kiwi ancestor arrived later by swimming. On the other hand, if the ratite ancestor was flying and flightlessness evolved several times among ratites, the kiwis can be assumed to have arrived later than the moas on New Zealand. Such an ancestral flying kiwi may have been related to the approximately 50- to 60-million-year-old flying birds with archaic palates that have been found in North America and Europe (1, 2). The idea that birds can lose flight capacity and change their morphology in adaption to a ground-dwelling existence over a short time span has support from island faunas on New Zealand (21) and elsewhere (30, 31). Further work on the rate of evolution of the 12S rRNA gene in birds is necessary to clarify this point.

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