

ing observations with the initiation of a reaction [for example, temperature jump and photochemical or electrochemical (9, 11) generation of a reactant], one can follow each molecule as it reacts to see how confined spaces and low concentrations affect reaction rates and equilibrium in order to compare those measurements with macroscopic measurements. The temporal resolution of the open-shutter experiment can be further improved with higher speed A/D cameras (4 MHz is commercially available) or by imaging narrow subframes. If the frame rate is increased to >10 kHz (16), then movies can be taken to construct trajectories (14, 15) in free solutions.

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15. After submission of our work, a related paper appeared [R. M. Dickson, D. J. Norris, Y.-L. Tzeng, W. E. Moerner, *Science* **274**, 966 (1996)]. By correlating the fluorescence spots in consecutive images, Dickson *et al.* followed molecular diffusional motion by constructing trajectories of motion. The images were recorded every 0.7 s, so only highly restricted motion, such as that found in certain pores of gel matrices, could be studied. Because evanescent-wave excitation was used, the observed intensities could be used to estimate the z position of the molecule, assuming there were no orientational or environmental effects on the spectroscopic properties. The molecules were not monitored continuously, so photobleaching could not be distinguished from movement away from the evanescent field. To study dynamics in free solution, as is demonstrated in our study, one needs a faster temporal response, the ability to observe the molecules continuously, and a way to decouple diffusion from photodecomposition.
16. The diffusion time  $t$  of R6G ( $D_{\text{R6G}} = 2.8 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ ) (7) for a diffusion distance  $\Delta$  of 0.2  $\mu\text{m}$  (one edge of a pixel) is 0.1 ms based on random-walk theory,  $\Delta = (2Dt)^{1/2}$  [L. B. Anderson and C. N. Reilly, *J. Chem. Educ.* **44**, 9 (1967)].
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## Mass Survival of Birds Across the Cretaceous-Tertiary Boundary: Molecular Evidence

Alan Cooper\* and David Penny

The extent of terrestrial vertebrate extinctions at the end of the Cretaceous is poorly understood, and estimates have ranged from a mass extinction to limited extinctions of specific groups. Molecular and paleontological data demonstrate that modern bird orders started diverging in the Early Cretaceous; at least 22 avian lineages of modern birds cross the Cretaceous-Tertiary boundary. Data for several other terrestrial vertebrate groups indicate a similar pattern of survival and, taken together, favor incremental changes during a Cretaceous diversification of birds and mammals rather than an explosive radiation in the Early Tertiary.

The Cretaceous-Tertiary (K-T) boundary, 65 million years ago (Ma), was a key episode in recent vertebrate history, marking the end of the dinosaur era and the rise to predominance of mammalian and avian taxa. This boundary is associated with both an extraterrestrial impact directly at the boundary and periods of flood-basalt volcanism (1). The biological consequences of these physical events are poorly understood and controversial (2), and several hypotheses have emerged in the absence of direct biological data.

It has been generally thought that Cretaceous avian and mammalian lineages also suffered a mass extinction and that a period of rapid adaptive radiation followed in the Tertiary from a few, or perhaps only a single, surviving lineage (3). Alternative hypotheses are that most modern avian (4) and mammalian orders survived the K-T boundary or that several lineages survived, perhaps on different continents. The last hypothesis is supported by molecular studies indicating that there are a few deep diver-

gences in small sets of both birds and mammals (5, 6). To further analyze this issue, we combined paleontological and molecular data to examine the K-T boundary extinctions and estimate the times of divergence of modern avian orders.

Molecular data alone cannot be used to determine the extent of extinctions, but, by inverting the question and estimating the number of modern avian and mammalian lineages that survived the K-T boundary, it is possible to estimate some of the biological consequences of the physical events at the boundary. We used early Tertiary fossil records of seven avian orders (Fig. 1) together with DNA sequences from mitochondrial and nuclear genes of a wide variety of avian taxa (7) to obtain a minimal estimate of the number of extant avian lineages that crossed the K-T boundary. We obtained a total of 42 sequences (8) from a 390-base pair (bp) region of the mitochondrial ribosomal small subunit RNA (12S) for 16 avian orders and 16 sequences (9) of a 600-bp region of the single-copy nuclear *c-mos* proto-oncogene (10) for 10 avian orders (11). We concentrated on groups for which Early Tertiary fossil dates were available (Fig. 1).

Our primary method for estimating the divergence times of avian orders is by a quartet method that works in two stages. In the first step, DNA sequences are compared from pairs of related birds for which fossils provide a minimum divergence time (12). Pairs must be selected with care so that

A. Cooper, School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand, and Molecular Genetics Laboratory, National Zoological Park, Smithsonian Institution, Washington, DC 20008, USA.

D. Penny, School of Biological Sciences, Massey University, PO Box 11222, Palmerston North, New Zealand.

\*To whom correspondence should be addressed at Department of Biological Anthropology, Oxford University, Oxford OX2 6QS, UK. E-mail: alan.cooper@bioanth.ox.ac.uk

there is no ambiguity in the minimum time of their divergence. A penguin fossil date is sufficient to date a penguin-albatross divergence (because seabirds are not derived from penguins) but, for example, a fossil date is needed for each of the gull-tropicbird pair (gulls could be derived from tropicbirds, or vice versa). An average rate of evolution is then estimated for each pair.

The next step is to combine two distantly related pairs of birds into a quartet and use the evolutionary rates to estimate the minimum divergence time of the two pairs forming the quartet (Fig. 2). The evolutionary tree for the quartets is not in question, and many quartet comparisons can be made to increase the accuracy of results (13). The estimated divergence times are conservative because, if an error was made in choosing pairs of taxa—for example, if they were not closely related—then an erroneously fast evolutionary rate, and correspondingly recent divergence date, would be calculated as a result of the quartet comparison.

This method does not depend on any universal molecular clock for birds, and, because only two orders are compared at a time, it is relatively insensitive to any rate variation among avian orders. If one order (say, galliforms) was evolving faster than average, then this method would give a more recent estimate for the time of overall divergence of birds. Any rate variation among orders is expected mainly to increase

the variance of the estimate. The quartet method only assumes that any changes in evolutionary rate are independent, that is, that different avian orders do not all speed up or slow down in unison. The use of two values for each pair also increases the accuracy (13) and, because only one parameter from each quartet is estimated, the method is expected to be well within the zone of accuracy (13) for sequences of this length.

The distribution of divergence dates estimated from quartet comparisons of the 42 12S sequences by themselves, and 16 combined 12S and *c-mos* sequences (Fig. 3), supports an Early Cretaceous date for the earliest divergence of modern avian orders. No quartet gives a divergence date after the K-T boundary. Indeed, the combined data set gives no estimate less than 100 Ma.

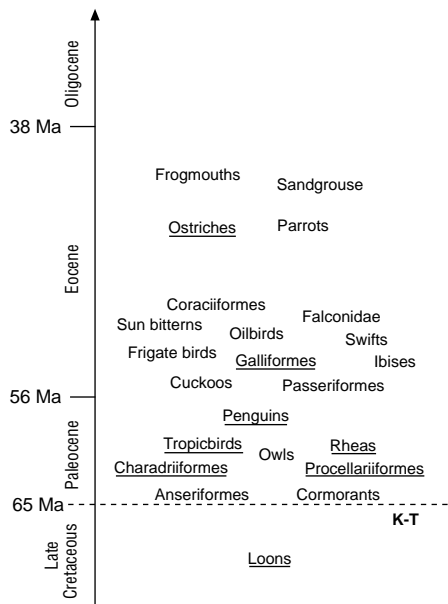
These results support an Early Cretaceous origin for modern birds as a whole but do not distinguish whether several or many lineages survived the K-T boundary. To investigate this issue further, we used the paleontological data to calibrate divergence times within a molecular phyloge-

netic tree. The tree (Fig. 4) is for 15 taxa for which both nuclear (*mos*) and mitochondrial (12S) sequences (a total of 952 nucleotides) were available. In overall topology the tree is independent of a tree-building method, and we constructed trees that were similar, using several models that assume stable nucleotide compositions as well as a model that does not (14). Standard bootstrap analysis shows that the basal divergences within the tree are well supported (Fig. 4).

In estimating divergence dates, the general robustness of the tree is more important than the exact topology, so we also measured the tendency and extent of lineages to change position in the tree (Fig. 4). We used standard bootstrap values to evaluate local rearrangements about internal branches of the tree to see how often taxa occurred in their given position or within one neighboring branch (15). Such nearest neighbor bootstrap values indicate the degree of support for a tree if minor local variations in taxon placement are permitted. Although the average value of the standard bootstrap

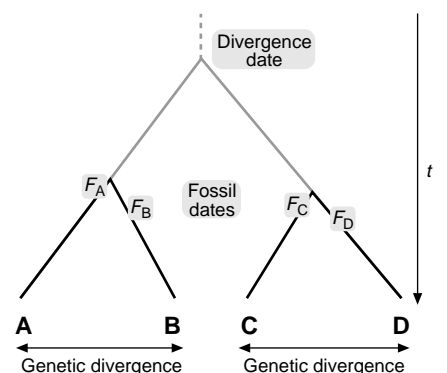
**Table 1.** Number of extant avian lineages estimated to cross the K-T boundary. Each of the 12S sequences was added individually to the log-determinant tree to obtain the branch point and corresponding divergence date relative to the K-T boundary.

Avian order	Common names	Number of lineages
Ratites	Rhea, ostrich, moa	3
Tinamiformes	Tinamou	1
Galliformes	Chicken, chachalaca	2
Anseriformes	Duck	1
Psittaciformes	Grey parrot, bluebonnet, kakapo	3
Pelecaniformes	Tropicbird, frigate bird	2
Charadriiformes	Gull	1
Passeriformes	Wren	1
Strigiformes	Owl	1
Falconiformes	Osprey	1
Threskiorniformes	Ibis	1
Gruiformes	Rail	1
Gaviiformes	Loon	1
Podicipediformes	Grebe	1
Procellariiformes	Albatross, shearwater	2
Total		22



**Fig. 1.** Earliest fossil records of modern bird orders (31, 32). Underlined taxa were used for calibrations (33). Large-bodied, or aquatic, taxa, which have increased chances of fossilization, are well represented in the Late Cretaceous–earliest Tertiary fossil record, suggesting that the delayed appearance of other orders may be a result of biases during fossilization processes.

**Fig. 2.** The divergence time of a quartet can be estimated from the calibrated evolutionary rates of two distantly related pairs of taxa. In this way the tree uniting the four taxa is not in question, and no assumptions need to be made about rate constancy across bird lineages because only two orders are being compared at a time. For example, if A–B and C–D represent the quartet rhea–ostrich and loon–shearwater, they show 57 and 63 observed substitutions between the sequences of A and B and of C and D, respectively, and another 38 on the root between the two pairs. Because each pair has fossil taxa ( $F_{A-D}$ ) within the Late Cretaceous–Paleocene, the common ancestor is expected to lie well within the Cretaceous. The estimated divergence dates are expected to be conservative because the discovery of either older fossils or more closely related sequences within a pair would lead to slower evolutionary rate estimates, and consequently older divergences.



analysis is 72% (SD, 14%), the average value of the nearest neighbor bootstrap is 90% (SD, 7.7%), indicating that the general tree topology is well supported.

The tree is generally consistent with other molecular phylogenies (16) and supports the estimated divergence dates from the quartet results (Fig. 3). The tree shows that the ratite and galliform lineages diverged early, followed later by the parrots, and that together these groups are sister taxa to a major clade including many other orders. The tree also identifies the penguin, loon, and seabird clade as being derived within birds, in agreement with other biochemical and molecular data (16). This result is important because, if all other orders are deeper in the tree than are the seabirds, then their times of divergence must be correspondingly older than those of the penguin and loon fossils. The owl and passerine have a significantly faster evolutionary rate than other taxa we examined, but these taxa did not affect the quartet analysis because no pair existed for either of them within the data sets, and consequently they could not be used.

We used this well-supported tree to determine the phylogenetic position of taxa for which only 12S sequences were available, by adding each taxon individually into the data set and determining its optimal position (Fig. 4). A neighbor-joining tree of all 42 12S sequences using log-determinant distances supports the conclusions about divergence times. Of the 42 lineages for which we have sequences, 22 are estimated to have Cretaceous origins (Table 1). However, for many orders of birds we either do

not have data or have just a single representative, although the order may contain several lineages that are pre-Tertiary, such as Passeriformes or Anseriformes. Consequently, this number is likely to be a considerable underestimate.

The analysis demonstrating that many lineages of birds survived the K-T boundary appears robust. The calibration points are close in time to the events being examined, and the divergence date estimates are independent of any individual fossil identification and date, do not rely on a universal avian molecular clock, and are independent of differing nucleotide compositions. The estimated Cretaceous divergences are supported by phylogenetic analyses anchored by seabird fossils, which show that other lineages, not derived from seabirds, must diverge earlier than 58 to 70 Ma.

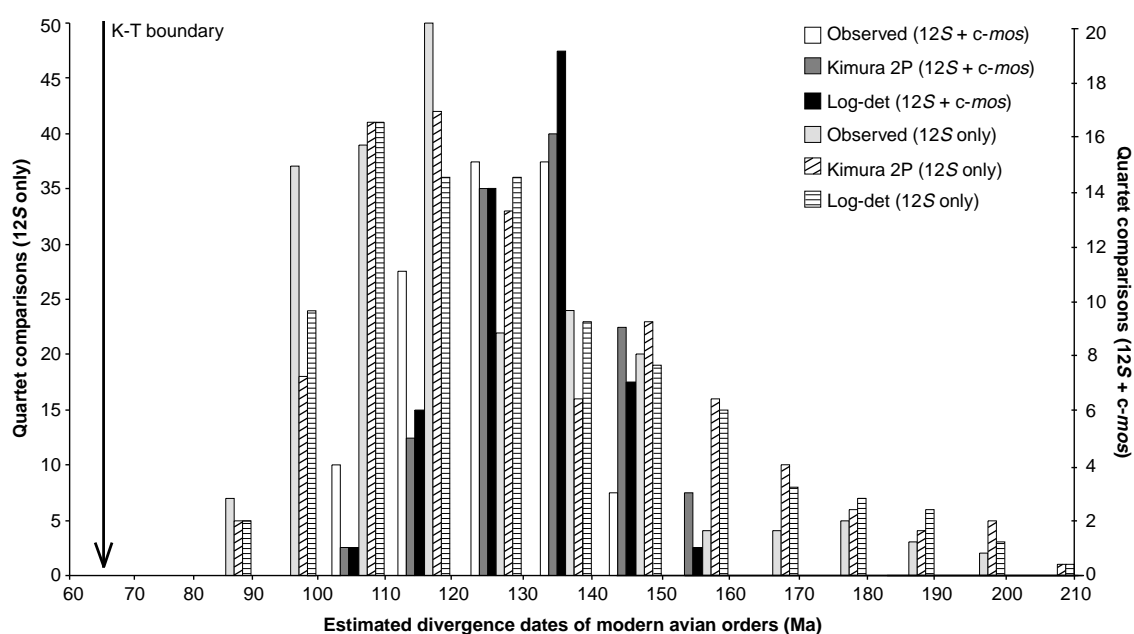
Recently, molecular data from three basal and one derived avian order were calibrated with a single Late Paleozoic (310 Ma) fossil date and were found to diverge in the Cretaceous (6). This result depends on the assumption that evolutionary rates were constant over the period analyzed (310 million years) but shows that divergence estimates for the modern bird orders are concordant whether a single Late Paleozoic date or many Early Tertiary calibration points are used.

Our hypothesis regarding Cretaceous origins of modern avian orders also appears to hold for some other groups of terrestrial vertebrates. Long mammalian mitochondrial and nuclear sequences (5, 17) can be calibrated with Early Tertiary mammal fossils (18). Using these calibration points and

currently available data, we estimate that at least 20 mammalian groups predate the K-T boundary. These include rodents (three lineages), edentates (two), lagomorphs (one), insectivores (three), ungulates (one), perisodactyl-carnivores (one), primates (two), elephant-hyraxes (one), aardvarks (one), elephant shrews (one), pangolins (one), marsupials (two), and monotremes (one). The avian and mammalian data can be combined with molecular data (19) and fossil records (20) of other terrestrial vertebrates (amphibians and later groups). The fossil record permits identification of lineages that survived the K-T boundary but subsequently became extinct. Using this combined data set, we estimate that a minimum of 100 terrestrial vertebrate lineages survived the end-Cretaceous extinctions. These results imply that the end-Cretaceous dinosaur extinctions were highly selective.

A mass extinction of birds at the K-T boundary would mean that most modern bird orders, with body plans as diverse as ratites, penguins, and passerines, evolved in approximately 5 to 10 million years after the K-T boundary. Such explosive rates of morphological evolution are difficult to reconcile with other measurements of avian morphological evolution in the Tertiary, where conservative and consistent rates have been noted (21). The required rates would be orders of magnitude faster than the most rapid radiations observed in the Late Tertiary, such as those of island taxa on Hawaii and Galápagos. The Hawaiian honeycreeper radiation is well known for diverse plumage and beak structures and is thought to have evolved during the last 5

**Fig. 3.** Estimated divergence dates for modern birds (in periods of 10 Ma) from 48 and 221 quartet comparisons of the combined and 12S data sets, respectively. The three sets of results are for sequences uncorrected for multiple changes, and corrected by the Kimura 2-parameter (2P) and log-determinant transformations (14). Many combinations of sequences and fossil dates are used, but all estimates are well before the K-T boundary at 65 Ma. The larger 12S data set of 42 taxa permits considerably more quartet comparisons, but many probably do not pass through the earliest possible divergence point within modern birds. Consequently, the distribution of estimates from the 12S data set is slightly younger and more variable than that for the combined data set.



million years (22). However, throughout this radiation the postcranial body plan shows comparatively minimal amounts of morphological evolution (23). On the other hand, if an Early Cretaceous divergence date is accepted for modern avian orders, standard microevolutionary mechanisms seem sufficient to account for the macroevolutionary trends observed.

The paucity of identifiable modern avian and mammalian orders in the Cretaceous fossil record is problematic. Our results are supported, however, by some recent fossil discoveries of Early Cretaceous ornithurine (modern) birds and advanced stages of avian and mammalian evolution in the Late Cretaceous and Early Tertiary (18, 24). In addition, recent analyses of fossil data corrected for paleontological and taxonomic biases indicate that most of the end-Cretaceous extinctions were marine (25). Consequently, the ecological impact on terrestrial systems may not have been cataclysmic, at least on a global scale.

The early fossil record of modern avian and mammalian taxa has historically been strongly biased toward finds in the Northern Hemisphere (26, 27) both because of the

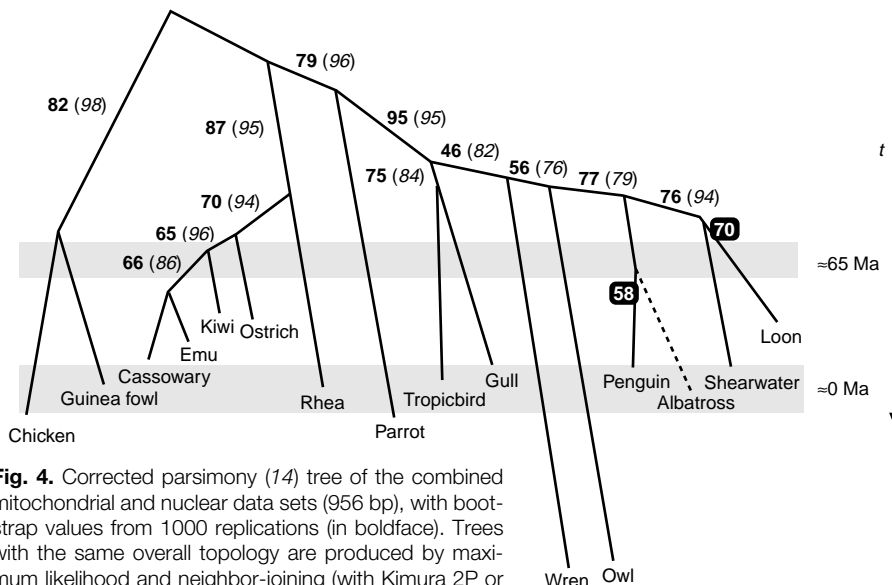
number and location of researchers and because of the belief that Laurasia was the center of terrestrial vertebrate evolution (28). The Laurasian hypothesis was reasonable when it appeared that avian and mammalian orders diverged in the Early Tertiary, when Gondwana had long been fragmented. However, our data and recent geological studies (29), which suggest that Gondwana presented fewer fossilization opportunities during this period, indicate that this hypothesis should be reexamined. Gondwana has long been acknowledged as a major source of flowering plant evolution (30), and the phylogeographic distributions of modern avian orders support a similar pattern (31). The earliest fossil records or basal lineages of many avian taxa are Gondwanic: ratites, galliforms, anseriforms, parrots, pigeons, passerines, loons, and penguins. It is possible that the relatively small number of Late Cretaceous avian and mammalian fossil deposits (26, 27), together with a geographic bias in scientific attention, may help explain the apparent schism between paleontological and molecular data.

The traditional view of an end-Cretaceous mass extinction of terrestrial verte-

brates relies heavily on negative evidence, largely from the Northern Hemisphere fossil record. In contrast, molecular data provide hard evidence for a Cretaceous diversification of modern mammals and birds, compatible with standard gradualistic mechanisms, followed by a mass survival across the K-T boundary.

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7. DNA was extracted from tissue and feather samples of a grebe (*Aechmophorus occidentalis*), two loons (*Gavia immer*, *G. arctica*), penguin (*Eudyptes schlegeli*), two albatrosses (*Diomedea epomophora*, *D. exulans*), petrel (*Pterodroma heraldica*), three shearwaters (*Calonectris diomedea*, *Puffinus griseus*, *P. pacificus*), frigate bird (*Fregata minor*), tropicbird (*Phaethon rubricauda*), gull (*Larus heermanni*), eagle (*Haliaeetus leucogaster*), osprey (*Pandion haliaetus*), guinea fowl (*Numida meleagris*), chachalaca (*Ortalis vetula*), ibis (*Plegadis falcinellus*), two tinamous (*Crypturellus tataupa*, *Northura maculosa*), parakeet (*Psittacula derbiana*), and two owls (*Strix varia*, *Ninox novaeseelandiae*); from bone of macaw (*Ara militaris*) and rail (*Rallus philippinensis dieffenbachii*); and from blood samples of three parrots (*Psittacus erithacus*, *Northelia haematogaster*, *Strigops habroptilus*) and a Fiordland crested penguin (*Eudyptes pachyrhynchus*) as described [A. Cooper and R. A. Cooper, *Proc. R. Soc. London Ser. B* **261**, 293 (1995)].
8. A 390-bp region of the third domain of the mitochondrial 12S gene was amplified by the polymerase chain reaction (PCR) from the above 29 taxa and sequenced as described by A. Cooper and R. A. Cooper [see (7)]. We obtained 12S sequences of chicken, mallard, Japanese quail, a New Zealand wren, a tinamou, and eight ratite taxa from GenBank (accession numbers X52392, X55530, X57245, X67626, X67630-35, X67637-38) and FTP sites (11). We aligned sequences (positions 1754 through 2147 of the chicken sequence) with the published human, mouse, and *Xenopus* sequences [J.-M. Neefs, Y. van de Peer, L. Hendriks, R. De Wachter, *Nucleic Acids Res.* **18**, 2237 (1990)] and 353 homologous positions were identified among the avian taxa by means of a revised avian 12S secondary structure model [R. E. Hickson *et al.*, *Mol. Biol. Evol.* **13**, 150 (1996)]. The effective sequence length (number of sites free to vary) was estimated to be 156 from variation within the avian taxa.
9. We amplified and sequenced a 690-bp region of *c-mos*, positions 183 through 789 of the published chicken sequence (12), from *Gavia arctica*, *Eudyptes pachyrhynchus*, *Puffinus griseus*, *Phaethon rubricauda*, *Larus heermanni*, *Strix varia*, *Psittacula derbiana*, and *Numida meleagris*; we did the same from *Rhea pennata*, *Struthio camelus*, *Casuaris casuaris*, *Dromaius novaehollandiae*, *Apteryx australis*, and *Acanthisitta chloris* using previously isolated DNA [A. Cooper *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8741 (1992); see A. Cooper and R. A. Cooper in (7)] by PCR with the following primers: 944, 5'-GCCTGGT-GCTCCATCGACTGG-3'; 1192, 5'-CCTCGGCAC-



**Fig. 4.** Corrected parsimony (14) tree of the combined mitochondrial and nuclear data sets (956 bp), with bootstrap values from 1000 replications (in boldface). Trees with the same overall topology are produced by maximum likelihood and neighbor-joining (with Kimura 2P or log-determinant distances), although the order of the owl, wren, tropicbird, and gull varied, and the kiwi and ostrich reversed positions. All these methods compensate for unequal rates of evolution, and, in addition, the log-determinant analysis allows unequal base compositions between lineages. The branch lengths are from the neighbor-joining log-determinant analysis and are drawn proportional to evolutionary distance. The values in italics represent nearest neighbor bootstrap values, giving the percentage of times an internal branch rearrangement occurs (for example, the ratite and galliform lineages are joined in 79% of the standard bootstrap replications with the parrot lineage as a sister taxon, whereas the nearest neighbor bootstrap value indicates that either this situation or one in which the parrot lineage combines with the ratite or with the galliform ancestor occurs in 96% of the replications). The nearest neighbor bootstrap values indicate that the general topology of the tree is robust. When the tree is rooted with amphibian (*Xenopus*) or mammalian (human or mouse) sequences, the root falls on one of the three basal lineages: galliform, ratite, or parrot, depending on the phylogenetic model used. Taxa represented only by 12S sequences (for example, the albatross) were individually added to this tree to determine their phylogenetic position, as shown (dashed line). Because nearly all divergences occur earlier in the tree than the date constrained by the penguin and loon fossils (shaded values), these divergences occur in the Cretaceous.

- TATCATCATGGA-3'; 1341, 5'-GCCCTGTGAGT-GAAGAAAGGC-3'; and 1550, 5'-GCAATGAGTA-GATGCTGCT-3'. We followed the procedure in (7), except that the primer annealing temperature was 58°C, and second-round amplification was performed with both primers at 0.1 μM. The primer numbering refers to the position of the 3' base in the published chicken *c-mos* sequence (12). We sequenced products using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed them on an Applied Biosystems 373A DNA sequencer. The sequences were aligned with the human, mouse, *Xenopus*, and chicken *c-mos* sequences (12), and 599 homologous positions were identified (positions 186 through 788 of the chicken sequence). The effective sequence length was estimated to be 250 from the number of leucine and third codon positions and variation observed in the 10 avian orders.
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  11. Sequences are available from GenBank, accession numbers U88000–88028 (for 12S sequences) and U88417–88430 (for *c-mos* sequences), and both aligned data sets are available on World Wide Web site <http://www.massey.ac.nz/~wwplbio/research/farside/programs.htm>
  12. When the phylogenetic relation of a taxon is uncertain (for example, tropicbird), we formed pairs of taxa using the closest genetic outgroup found within the data set (gull, in this case). The pairs used were: rhea versus tinamou, moa, ostrich, emu, cassowary, kiwi; ostrich versus emu, cassowary, kiwi; cracid versus guinea fowl, chicken; chicken versus guinea fowl; penguin versus albatross; loon versus shearwater; and gull versus tropicbird.
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## Muscular Force in Running Turkeys: The Economy of Minimizing Work

Thomas J. Roberts,\* Richard L. Marsh, Peter G. Weyand, C. Richard Taylor†

During running, muscles and tendons must absorb and release mechanical work to maintain the cyclic movements of the body and limbs, while also providing enough force to support the weight of the body. Direct measurements of force and fiber length in the lateral gastrocnemius muscle of running turkeys revealed that the stretch and recoil of tendon and muscle springs supply mechanical work while active muscle fibers produce high forces. During level running, the active muscle shortens little and performs little work but provides the force necessary to support body weight economically. Running economy is improved by muscles that act as active struts rather than working machines.

Running is a bouncing gait in which mechanical energy is absorbed to slow and lower the body in the first half of a step and released to lift and accelerate the body in the second half of a step. Although some of this work can be provided passively by elastic energy storage in tendons (1, 2), active muscles must provide the force necessary to support the body and maintain tension on tendon springs. Differences in the energy cost of running across animal size and speed are proportional to the

cost of producing this force (3) but not to the rate at which mechanical work is performed (2). In vitro, muscles that contract isometrically (without shortening) perform no work but use metabolic energy and produce high forces. In contrast, a shortening contraction that maximizes mechanical work rate (power) produces only one-third the force of an isometric contraction, due to the characteristic force-velocity relation of skeletal muscle (4). This trade-off between work rate and force output suggests that animals could minimize the cost of producing force during running by operating active muscle fibers isometrically while the stretch and recoil of tendon springs provide the work of the bouncing body.

We used surgically implanted sonomicrometer crystals to measure fiber length and strain gauges to measure muscle force in the

T. J. Roberts, P. G. Weyand, C. R. Taylor, Harvard University, Museum of Comparative Zoology, Concord Field Station, Old Causeway Road, Bedford, MA 01730, USA. R. L. Marsh, Northeastern University, Department of Biology, 360 Huntington Avenue, Boston, MA 02115, USA.

\*To whom correspondence should be addressed at the Department of Biology, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA.

†Deceased.