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Tinamous and Moa Flock Together: Mitochondrial Genome Sequence Analysis Reveals Independent Losses of Flight among Ratites

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Abstract.—Ratites are large, flightless birds and include the ostrich, rheas, kiwi, emu, and cassowaries, along with extinct members, such as moa and elephant birds. Previous phylogenetic analyses of complete mitochondrial genome sequences have reinforced the traditional belief that ratites are monophyletic and tinamous are their sister group. However, in these studies ratite monophyly was enforced in the analyses that modeled rate heterogeneity among variable sites. Relaxing this topological constraint results in strong support for the tinamous (which fly) nesting within ratites. Furthermore, upon reducing base compositional bias and partitioning models of sequence evolution among protein codon positions and RNA structures, the tinamou–moa clade grouped with kiwi, emu, and cassowaries to the exclusion of the successively more divergent rheas and ostrich. These relationships are consistent with recent results from a large nuclear data set, whereas our strongly supported finding of a tinamou–moa grouping further resolves palaeognath phylogeny. We infer flight to have been lost among ratites multiple times in temporally close association with the Cretaceous–Tertiary extinction event. This circumvents requirements for transient microcontinents and island chains to explain discordance between ratite phylogeny and patterns of continental breakup. Ostriches may have dispersed to Africa from Eurasia, putting in question the status of ratites as an iconic Gondwanan relict taxon. [Base composition; flightless; Gondwana; mitochondrial genome; Palaeognathae; phylogeny; ratites.]

Modern birds have long been taxonomically divided on the basis of palatal characters (e.g., Huxley 1867) into Neognathae, which make up over 99% of all extant avian species, and Palaeognathae, which includes ratites and tinamous. Analyses of nuclear genes and complete mitochondrial (mt) genomes strongly support this primary avian division (e.g., García-Moreno and Mindell 2000; Hugall et al. 2007; Slack et al. 2007). The ratites, which are all flightless, are the most familiar palaeognaths and are generally large herbivores/omnivores. Extant members include the ostrich, rheas, emu, cassowaries, together with the recently extinct (post-human) moa of New Zealand and elephant birds of Madagascar. In addition, there are the smaller and primarily invertebrate-feeding kiwi. Bertelli and Porzecanski (2004) recognized 9 genera and 47 species of tinamou, all in South America. They are ground foraging birds that fly but are not considered strong flyers.

Although some early workers (e.g., Mayr and Amadon 1951) questioned ratite monophyly, recent morphological studies support tinamous and ratites being reciprocally monophyletic sister taxa but provide little consensus on affinities within ratites (see Lee et al. 1997; Livezey and Zusi 2007). Early molecular studies involving immunological distances (Prager et al. 1976), DNA–DNA hybridization (Sibley and Ahlquist 1990), and short DNA sequences (e.g., van Tuinen et al. 2000) are similar in recovering a ratite/tinamou division, while not clearly resolving relationships between ratite families, except for Casuariidae (cassowaries plus emu). Sequencing complete mt genomes, including several extinct moa, provided a substantial leap in statistical power for resolving ratite relationships (Cooper et al. 2001; Haddrath and Baker 2001). These papers appear to have founded an mt consensus among molecular studies on ratite phylogeny (see Fig. 1a), which has since been followed by numerous studies of molecular dating (e.g., Pereira and Baker 2006; Brown et al. 2008), biogeography (e.g., Sanmartín and Ronquist 2004; Karanth 2006), and phylogenetic inference (e.g., Paton et al. 2002).

Ratites, along with southern beaches (*Nothofagus*) and cichlid and galaxiid freshwater fish, are considered both to be quintessential Gondwanan taxa (e.g., Briggs 2003; Waters and Craw 2006) and to provide substantive evidence for vicariance models of biogeography (e.g., Cracraft 1974). Indeed, among these, only ratites are known to have been distributed across all the major Gondwanan landmasses. The early molecular studies of Prager et al. (1976) and Sibley and Ahlquist (1990) claimed good agreement with the vicariance hypothesis: Ostrich (Africa) basal, then Rheas (South America) as sister to the Kiwi (New Zealand), and Casuariidae (Australia-New Guinea).

The mt consensus (Fig. 1a) provides a challenge for interpreting Gondwanan biogeography. First, molecular estimates for the divergence between the Kiwi and Casuariidae (mean/point estimates from 45 Ma [Härlid et al. 1998] to 77 Ma [Pereira and Baker 2006]) postdate the separation of New Zealand and Australia, which began (and was initially rapid) before 80 Ma (Lawver et al. 1992). Paton et al. (2002) estimated older divergences for kiwi, although their results may be compromised by the *Emuarius* calibration date being increased

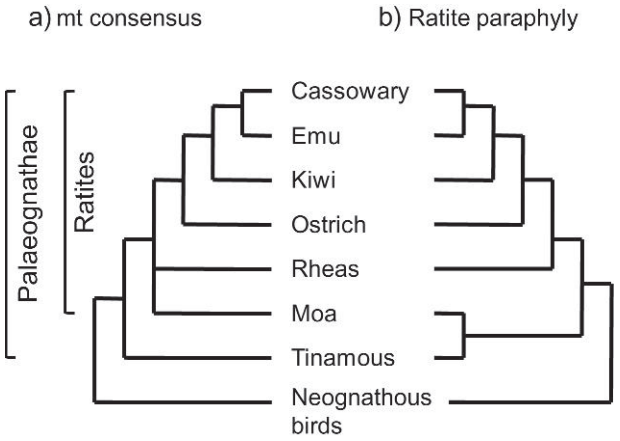

FIGURE 1. The "mt consensus" of palaeognath phylogeny (a) showing ratite monophyly and (b) the alternative topology of ratite paraphyly supported by our reanalysis of the mt data set of Cooper et al. (2001), in which a tinamou–moa grouping is favored.

by 40% without biological or geological explanation. Extended persistence of the Norfolk Rise and Lord Howe Ridge has been proposed to permit nonvolant (i.e., nonflying) dispersal between Australia and New Zealand, although these routes may have been submerged by 75 Ma (Cooper and Millener 1993).

A second biogeographic issue concerns the origins of African ratites. Geotectonic reconstructions show that Africa and South America were disconnected by at least 102 Ma (Veevers 2004), whereas fish fossil records indicate that open marine conditions in fact existed between these continents before 110 Ma (Maisey 2000). Explanations involving either vicariance or dispersal via the northern hemisphere both predict that the ostrich would be deep among ratites. Instead, rheas and moa fall to the base of the tree. Some geotectonic reconstructions (e.g., Hay et al. 1999) allow the possibility of the Kerguelen Plateau connecting Australia/Antarctica with Indo-Madagascar until around 80 Ma. This could have provided a staging post for ratite dispersals into Africa and Eurasia, whereas the ancestors of elephant birds remained on Madagascar. The presence of ostrich-like ratites in Early-Mid Tertiary Eurasia and flying palaeognathous birds (lithornithids) in Early Tertiary North America and Eurasia were cited in an alternative proposal for explaining discordance between ratite phylogeny and biogeography, namely that flight has been lost independently among ratites (e.g., Houde 1986).

The most serious challenge yet to ratite monophyly and the associated single origin of flightlessness and Gondwanan vicariance implications has come from the "Early Bird" Tree of Life project. In two recent papers (published after this work was first submitted) based on 20 or more nuclear loci, Hackett et al. (2008) and Harshman et al. (2008) find tinamous to be nested within ratites and the ostrich to be "basal" among extant palaeognaths, thus favoring ratite paraphyly over monophyly. Nevertheless, noncoding sequences that are often difficult to align dominate the "Early Bird" studies, and questions have been raised over the validity of other findings based primarily on the difficulty of getting good alignments of intronic sequences for deep avian divergences (see Morgan-Richards et al. 2008; Pratt et al. 2009). It is noteworthy here that partitioned maximum likelihood (ML) bootstrap support for tinamous grouping within ratites to the exclusion of the ostrich falls to 62% in Harshman et al. (2008) when only the exonic sequences are included. In light of this, reevaluation of the mt evidence is timely.

Identifying mt signal for tinamous grouping within ratites would lend important confirmation to the nuclear results. Equally, it is critical to understand why previous mitogenomic phylogenies have (apparently) incorrectly supported ratite monophyly, especially given the reliance on mt data for most ancient DNA studies and burgeoning mt genome availability from shotgun sequencing projects (e.g., Gilbert et al. 2007). We are also able to address a number of other questions the Early Bird studies leave unanswered: the sister group of the tinamous within ratites, the placement of the extinct moa and elephant birds, the timescale of palaeognath evolution, and statistical support for alternative scenarios for both dispersal and loss of flight among palaeognaths.

Previous analyses of palaeognath phylogeny based on molecular sequences have revealed a high rate of evolution among the tinamous relative to the ratites (e.g., Sibley and Ahlquist 1990; Paton et al. 2002). Hence, in the absence of other "long-branch" taxa among the palaeognaths, there is an expectation that the tinamous will tend to be attracted toward the deeper outgroup taxa (see Hendy and Penny 1989), so artifactually reinforcing ratite monophyly. Long-branch attraction artifacts depend on "unobserved" substitutions not being sufficiently accounted for and thus, the amount of parallel change being underestimated (see Felsenstein 1978). Modeling variation in substitution rates across sites (RAS) is critical in correcting for such unobserved substitution.

Ratite monophyly has been enforced in all previous molecular phylogenetic analyses that modeled rates-across-sites heterogeneity and included both tinamous and moa (e.g., Cooper et al. 2001; Paton et al. 2002; Pereira and Baker 2006). In order to reduce the influence of branch-length biases relative to earlier studies, we incorporated among-site rate heterogeneous models across separately modelled protein codon and RNA structural data partitions. Further to this end we have increased outgroup taxon sampling relative to previous studies and report newly completed mt genome sequences for 2 species of kiwi.

Our analyses strongly support moa grouping with tinamous and these together most likely being sister to a group that includes kiwi, cassowaries, emu, and elephant birds. The implication that the ostrich is the sister to all other extant palaeognaths is further supported by base frequency (BF) distance trees that were employed to examine the topological nature of base composition nonstationarity. We use relaxed molecular clock

methods to provide a temporal scale for this revised palaeognath phylogeny and consider its implications for independent origins of flightlessness and, in turn, for biogeography.

Material and Methods

Polymerase Chain Reaction and Sequencing

Two kiwi species were sequenced as part of this study. The brown kiwi (*Apteryx australis mantelli*) sequence is from the same specimen (K86) and DNA extraction as used in Cooper et al. (2001) and completes the mt genome reported therein. The little spotted kiwi (*Apteryx owenii*) sample was from the Otorohanga Kiwi House. Whole mitochondria were isolated from blood using red blood cell isolation followed by cell disruption, differential centrifugation, and DNase I digestion. The method is based on that of Higuchi and Linn (1995). Polymerase chain reaction (PCR) was used to confirm no nuclear DNA remained in the mt extraction.

Brown kiwi DNA was amplified in one 12-kb long-range product spanning the incomplete region from Cyt *b* to NADH1 using the Expand Long Template PCR System (Roche, Auckland, New Zealand). Both the long-range PCR product (brown kiwi) and mtDNA extraction (little spotted kiwi) were used as templates for subsequent PCR of short 1- to 2-kb overlapping fragments, using primers described in the supplementary appendix (available from http:/www.sysbio.oxforjounals.org/). This process is described in more detail in Gibb et al. (2007) and references therein. The complete mt genome of the little spotted kiwi is 17,020 bp long (GU071052) and the brown kiwi is 17,058 bp long (GU071057, AY016010). Both kiwi have the standard gene order found in all ratites.

Data Matrices

The data set includes complete mt genome protein, ribosomal RNA and transfer RNA coding sequences, totalling 14,190 nucleotides (upon exclusion of sequences with ambiguous homology, after alignment in Se-Al 2.0a9; Rambaut 1996). In addition to the new kiwi sequences, 12 other palaeognathous birds, 8 neognathous birds, and 2 crocodilians were sampled in the present study. These included great-spotted kiwi (*Apteryx haasti*, NC_002782), cassowary (*Casuarius casuarius*, NC_002778), emu (*Dromaius novaehollandiae*, NC_002784), giant moa (*Dinornis giganteus*, NC_002672), eastern moa (*Emeus crassus*, NC_002673), little bush moa (*Anomalopteryx didiformis*, NC_002779), greater rhea (*Rhea americana*, AF090339), lesser rhea (*Pterocnemia pennata*, NC_002783), ostrich (*Struthio camelus*, NC_002785), giant tinamou (*Tinamus major*, NC_002781), elegant crested tinamou (*Eudromia elegans*, NC_002772), tataupa tinamou (*Crypturellus tataupa*, AY016012), chicken (*Gallus gallus*, NC_001323), brush turkey (*Alectura lathami*, NC_007227), magpiegoose (*Anseranas semipalmata*, NC_005933), redhead duck (*Aythya Americana*, NC_000877), blackish oystercatcher (*Haematopus ater*, NC_003713), ruddy turnstone (*Arenaria interpres*, NC_003712), little blue penguin (*Eudyptula minor*, NC_004538), red-throated loon (*Gavia stellata*, NC_007007), American alligator (*Alligator mississippiensis*, AF069428), and caiman (*Caiman crocodilus*, NC_002744). Data sets and phylogentic trees are available from TreeBASE (SN4712).

Neognath birds and crocodilians are the closest living outgroups to palaeognaths. Furthermore, base frequency (BF) distances per variable site ($|A_i - A_j| + |C_i - C_j| + |G_i - G_j| + |T_i - T_j|$) from the palaeognaths for the combined protein-coding and RNA-coding alignment are smaller for the neognaths (average 0.088) and crocodilians (average 0.063) than for the non-archosaurs (average 0.136), green turtle, eastern painted turtle, blue-tailed mole skink, and iguana that were included in the studies of Harrison et al. (2004) and Slack et al. (2006).

Numerous studies have revealed compositional heterogeneity to be of particular concern for mitogenomic phylogenetics (e.g., Delsuc et al. 2003; Gibson et al. 2005). A recent examination of compositional heterogeneity among birds (Harrison et al. 2004) includes several relevant findings: 1) The influence of compositional heterogeneity on phylogenetic reconstruction was exacerbated among data partitions for which saturation has greatly eroded phylogenetic signal (e.g., third codon positions); 2) Compositional χ^2 tests are poor indicators of potential for phylogenetic bias and they are not comparable between data sets because their statistical power depends on factors such as the number of variable sites; and 3) Coding nucleotide data as purines and pyrimidies (RY-coding) was more efficient (in terms of phylogenetic signal retention) than using the amino acid sequence for excluding sources of compositional heterogeneity.

We follow the recommendation of Harrison et al. (2004) to RY-code protein third codon positions. Two metrics described in that paper and originally in Phillips et al. (2001) provide further justification for this RY-coding. First, the stemminess (the proportion of internal branch length contributing to tree length) of minimum evolution trees on *p*-distances inferred from the present data set third codon positions increases from 0.170 to 0.230 upon RY-coding. Simultaneously, relative composition variability (RCV) among third positions falls from 0.093 to 0.054. RCV is the average variability in composition between taxa; for nucleotides this is

$$\mathrm{RCV} = \sum_{i=1}^{n} (|A_i - A^*| + |T_i - T^*| + |C_i - C^*| + |G_i - G^*|)/nt,$$

A_i, T_i, C_i, and G_i are the frequencies of each nucleotide for the *i*th taxon; A*, T*, C*, and G* are averages across the t taxa; and n is the number of sites. Uninformative sites effectively dilute apparent nonstationarity so were excluded (along with gapped sites) from RCV calculations.

Lower stemminess indicates greater phylogenetic signal erosion, and as noted in Phillips and Pratt (2008),

this compounds the potential for higher composition variability to mislead phylogenetic reconstruction. The improvements in both RCV and stemminess upon RY-coding the third positions provide considerable encouragement. Nevertheless, the possible influence of remnant compositional heterogeneity on phylogenetic reconstruction is also examined using BF distance trees (see below).

Phylogenetic Analyses

Palaeognath phylogeny was inferred from the mt genome data set as a single concatenation or partitioned by structure (stem and loop sites) for the RNA-coding data and by codon positions for the protein-coding data. Under the Akaike information criterion (AIC), this partitioning scheme was preferred over concatenation and alternative gene or gene-by-codon partitioning schemes (see Table 1). Bayes factor analyses within Tracer v1.4 (Rambaut and Drummond 2007) on Bayesian inference (BI) analyses (see below for details) performed in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) further support the conclusions based on the AIC results.

Given concerns for the influence of the long tinamou branches, phylogenetic analyses were repeated without the tinamous and the relationships among the ratites examined for consistency. Substitution model categories for each partition were assigned according to ModelTest 3.6 (Posada and Crandall 1998) AIC recommendations. In each case, these were GTR+I+Γ_4 for standard nucleotide partitions and F81+I+Γ_4 (equivalent to CF87+I+Γ_4; Cavender and Felsenstein 1987) for the RY-coded mt third codon partitions.

BI (MrBayes 3.1.2) analyses were run with the full substitution model and branch-length rate multipliers unlinked among the protein codon and RNA structural partitions. Within this framework, the GTR+I+Γ_4 version of the doublet model was employed for RNA stem pairs. Three Markov chain Monte Carlo (MCMC) chains for 2 independent runs proceeded for 3,000,000 generations with trees being sampled every 2000 generations. The burn-in for each MrBayes run (250,000) ensured that $-\ln L$ had plateaued, clade frequencies had converged between runs, and estimated sample sizes for substitution parameter estimates were above 200 (using Tracer v1.4).

ML analyses were performed within PAUP*4.0b10. ML bootstrapping (500 replicates) applied heuristic searches to random starting trees for the data sets as single concatenations. Following Phillips and Penny (2003), the TN93 (Tamura and Nei 1993) substitution model was applied to these concatenations such that the transversions in the standard nucleotide and RY-coded data are effectively weighted equally. As an alternative to optimizing substitution parameters on a Neighbor-Joining distance tree (as per ModelTest), these were optimized on the maximum parsimony (MP) tree, employed in an ML heuristic search and reoptimized on the resulting tree for use in the bootstrap analysis. In order to ensure computational feasibility for these bootstrap analyses, clades that are uncontroversial in all recent molecular and morphological classifications and also received posterior probabilities of 1.00 in the Bayesian analysis were constrained. These include Alligatorinae, Aves, Galloanserae, Galliformes, Anseriformes, Charadriiformes, Rheidae, Apterygidae, Casuariidae, and Dinornithidae.

Support among alternative topologies was further examined with KH (Kishino and Hasegawa 1989) and approximately unbiased (AU; Shimodaira 2002) tests, using the RELL method (100,000 replications) within CONSEL (Shimodaira and Hasegawa 2001). The AU test is related to the SH test (Shimodaira and Hasegawa 1999) and has been developed in order to overcome tree selection biases that affect the latter test when multiple topologies are being simultaneously compared.

The ML significance tests were applied with the sequences treated as separately modeled process partitions among codon positions (1, 2, 3) and RNA structures (stems, loops). Partitioning the data allows for more accurate models of sequence evolution that address differential influences on mutation and selection across the sequence (e.g., Yang 1996; Caterino et al. 2001; Buckley et al. 2002). Substitution model categories again followed the ModelTest AIC recommendations for both the standard nucleotide-coded partitions (GTR+I+Γ_4) and the RY-coded mt third codon partitions (CF87+I+Γ_4). All substitution parameters and branch lengths were ML optimized for each partition, for each tree hypothesis.

TABLE 1. Evaluation of partitioning using the AIC

Partitioning scheme[a]	df[b]	$-\ln L$	AIC	Bayes factor[c]
1a. All data (concat)	55	−95, 137.51	190, 385.02	382.7
1b. Ptn + RNA (par)[d]	110	−94, 232.91	188, 685.82	
2a. RNA (concat)	55	−18, 559.93	37, 229.86	116.3
2b. Stems + loops (par)[d]	110	−18, 261.55	36, 743.10	
3a. Ptn (concat)	55	−75, 672.98	151, 455.96	3005.2
3b. Ptn (par-gene)	715	−74, 564.38	150, 558.76	2574.5
3c. Ptn (par-codon)[d]	158	−72, 672.06	145, 660.12	
3d. Ptn (par-gene-by-codon)	2054	−70, 817.28	145, 742.56	

[a]Data are partitioned into (1) protein (Ptn) and RNA, (2) stems and loops for the RNA alone, and (3) individual genes, codon positions, or codon positions for each gene, for protein data alone.

[b]ML analysis (in PAUP* 4.0b10) of the 24-taxon data set provides 55 degrees of freedom (df) for each GTR+I+Γ modeled partition and 48 df for each CF87+I+Γ modeled RY-coded partition (third codon positions).

[c]ln Bayes factor differentials from the favored hypothesis except for the extremely parameter-rich 3D scheme, for which independent MrBayes runs of 100,000,000 generations showed no indications of converging or reaching stationarity.

[d]The favored scheme in each evaluation.

BF Distance Trees

Minimum evolution BF distance trees were constructed in PAUP* from matrices of pairwise BF

distances to assess the potential for compositional bias to affect phylogenetic inference. The basic idea is to compare BF distance tree-length differences between alternative topologies. In this way, Phillips and Penny (2003) showed that composition bias likely explains incorrect rooting of the mammalian tree in earlier mt genome studies. BF distances are half the sum of absolute frequency differences between taxon pairs for each nucleotide category. So the pairwise BF distance between taxa i and j is

$$\text{BF distance} = (|Ai - Aj| + |Ti - Tj| + |Ci - Cj| + |Gi - Gj|)/2,$$

Ai, Ti, Ci, and Gi and Aj, Tj, Cj, and Gj are the frequencies of each nucleotide for the ith and jth taxa, respectively. Dividing by 2 is necessary for minimum evolution (ME) distances between BF distance trees to be comparable with ME differences on standard (absolute) distance trees. This is because a substitution at a site in taxon i that previously had the same base as for taxon j will result in 1 unit of standard distance but 2 units of BF distance. Parsimony-uninformative characters were excluded from BF distance calculations as these cannot explain ME differences between standard distance trees. For all the ME trees, any negative branch lengths were treated as absolute values for computing tree length.

Molecular Dating

We estimated a timescale for palaeognath evolution using BEAST v.1.4.8 (Drummond and Rambaut 2007) with the 24-taxon data set partitioned as per the phylogenetic analyses. Among molecular dating programs BEAST is unique in incorporating a combination of characteristics that are desirable for analysis of the present data set. These include 1) separate GTR+I+Γ model allocation across the protein codon and RNA structure data partitions, including the equivalent model for the RY-coded third codon positions, 2) soft-bound calibration prior distributions, and 3) relaxation of the molecular clock without assuming rate correlation among branches.

An uncorrelated relaxed clock model was used with rates among branches distributed according to a lognormal distribution, which can provide greater flexibility than the exponential distribution option (Drummond et al. 2006). Note that a strict clock was rejected by a likelihood ratio clock test (using ML in PAUP*) at $P < 0.0001$, and in BEAST the null hypothesis of no rate autocorrelation among branches could not be rejected even at $P \leqslant 0.2$. Five independent runs totaling 90,000,000 MCMC generations ensured estimated sample size values >100 (as estimated in Tracer v1.4; Rambaut and Drummond 2007) for all node height, prior, posterior, $-\ln L$, tree, and substitution parameters. Chains were sampled every 5,000$^{\text{th}}$ generation after burn-ins of 2,000,000 generations.

Difficulties with calibrating molecular dating analyses for the Palaeognathae have not been fully appreciated. Two "internal" ratite calibration dates have commonly been used. One is geotectonic, the divergence between Australia and New Zealand, at ≈82 Ma (Cooper et al. 2001). The present finding of a tinamou–moa grouping undermines the basis of this calibration, which assumes that the divergence between moa and Casuariidae from a flightless ancestor predates the geotectonic divergence. The second internal calibration, the divergence between cassowaries and emus being >25 Ma, is not influenced by the finding of multiple losses of flight among ratites. However, this relies on accepting that the Late Oligocene/Early Miocene casuariiform, *Emuarius*, shares a closer phylogenetic relationship with emus than with cassowaries (Boles 1992). The tibiotarsus (lower leg bone) suggests *Emuarius* affinities with emus. This hypothesis depends partly on the assumption that the most recent common ancestor (MRCA) of Casuariidae was more cassowary-like such that skull and femur similarities between *Emuarius* and cassowaries are symplesiomorphic.

In order to provide temporal calibration, we have employed prior height distributions for 5 nodes external to ratites. We follow Barnett et al. (2005) in our usage of calibration bounds. The minimum marks the first appearance of a generally agreed upon member of the crown group, and the maximum covers the time back until relatively well-sampled fossil assemblages in potential geographic regions of origin contain no putative crown group members but contain stem members or ecological equivalents. Selection of uniform, normal, or lognormal distributions for calibration priors follows Ho and Phillips (2009).

Root (Archosauria): Normal distribution, 95% range from 235 to 250 Ma (Benton and Donoghue 2007).

Aves: Normal distribution, 98% range from 66 to 121 Ma. The normal 98% range acknowledges that both the minimum and the maximum are extremely conservative, and prior expectations for the actual value to be well within the given range are higher than for the other nodes. The minimum is based on *Vegavis* (Clarke et al. 2005) which branches at least 3 nodes internal to the root of modern birds. The maximum is based on the age of the younger of the avian bearing beds within the Jehol biota (Zhou 2006), from which (and before) only Enantiornithes and other "primitive" birds are known. Although the maximum allows for Early Cretaceous birds such as *Gansus* (110 Ma; You et al. 2006) being within the avian crown group, it is far more conservative than the 86 Ma of Benton and Donoghue (2007).

Galloanserae: Uniform distribution, range from 66 to 86 Ma (Benton and Donoghue 2007).

Seabirds (penguin vs. loon): Lognormal distribution, hard minimum 61 Ma (based on the penguin, *Waimanu*; Slack et al. 2006). Mean at 65.5 Ma and 97.5% soft maximum at 74 Ma, respectively, reflect expectations for a K/T boundary radiation after the extinction of numerous avian stem seabirds (Feduccia 1996) and the possibility of seabirds evolving in the Southern Hemisphere during the relative hiatus in that region's late Campanian to late Maastrichtian fossil record.

Alligatorinae: Lognormal distribution, hard minimum (64 Ma), mean expectation (70 Ma), and 97.5% soft maximum (80 Ma) follow Brochu (2004).

Two internal palaeognath calibrations were also included as uniform priors for a second BEAST analysis.

Casuariidae (emu vs. cassowary): Uniform distribution, range from 25 to 35 Ma (see Haddrath and Baker 2001; Phillips 2009).

Rheas versus Casuariidae/kiwi/tinamou–moa: Uniform distribution, range from 56 to 83 Ma. The minimum age is provided by *Diogenornis* (Alvarenga 1983), and the maximum covers the absence of even putative members of this clade in well-sampled Campanian faunas from South America and the Northern Hemisphere.

These internal palaeognath calibrations were not employed in our primary analysis because of the reservations stated above regarding *Emuarius* as a crown cassuariid and also some uncertainty remains over the status of *Diogenornis* as a stem rheiid.

Ancestral State Reconstruction

Histories for dispersal and loss or gain of flight among palaeognaths were inferred under MP and ML criteria in PAUP* and BayesTraits (Pagel and Meade 2006), respectively. The 24 taxa from the mitogenomic data set were coded as flighted or flightless. Character coding for the dispersal analysis was more complicated. First, in order to help reduce geographic sampling biases, regional assignments were considered at the family level, rather than the species level. This did not affect coding for the palaeognaths but resulted in several outgroup taxa being polymorphic. Second, due to landmasses shifting, the rate matrix should not be expected to be homogenous across the tree, particularly given the break-up of South Gondwana (South America, Australia–Antarctica, New Zealand) from about 80 Ma. We take this nonhomogeneity into consideration by using a nested design for our ML dispersal analyses.

For the overall tree, regions were coded as Northern Hemisphere, Africa, and South Gondwana. Within palaeognaths, the most inclusive clade originating after 80 Ma has modern members only on the former South Gondwana landmasses and its MRCA was assigned to South Gondwana in the overall tree ML analysis with $P > 0.99$. Hence, further analyses were performed on the South Gondwana clade (Rheidae, Tinamidae, Dinornithidae, Apterygidae, Casuariidae) assigned to South America, Australasia, and New Zealand. This nested design also has the benefit of reducing parameterization relative to having a single analysis with 5 regional character states. State transitions were symmetrically reversible in the dispersal analyses, in accord with likelihood ratio tests rejecting the asymmetrical alternatives at $P < 0.05$.

In considering heterogeneity in dispersal probabilities over time, our analyses have a partial analog with the more sophisticated dispersal–extinction–cladogenesis (DEC; e.g., Ree and Smith 2008). However, our method has much reduced parameterization (important for smaller data sets). Second, BayesTraits assumes that ancestral individuals are localized to one "area state," as befitting ratite mt evolution over timescales of nearly 100 Ma. In contrast, DEC is specialized for population biogeography and allows ancestors to be distributed over 2 or more "area states" for substantial proportions of lineage time even when organisms at all the tips are localized.

The BayesTraits analyses used the multistate ML option with 2 and 3 state categories, respectively, for flight gain/loss and regions, with characters evolving along the BEAST (median node height) dated tree. This design gives us the options of including extinct taxa and artificially altering sampling strategies, while maintaining comparability among these treatments (i.e., negating differential influences from priors and integration across alternative phylogenetic trees).

RESULTS

Preliminary Phylogenetic Analyses

As noted in Introduction, the mt consensus on ratite phylogeny (Fig. 1a) is based on analyses for which either ratite monophyly has been enforced or rates-across-sites variation has not been allowed for. Cooper et al. (2001) was the first of the complete mt genome studies to include the extinct moa. We reemployed their data set and ML methodology (see Appendix) and only by enforcing ratite monophyly were we able to replicate their tree, in which rheas are sister to all other ratites (consistent with Fig. 1a). Relaxing that monophyly enforcement results in the tinamous shifting from a basal position among palaeognaths to be sister to moa (Fig. 1b). This alternative tree is 15.365 $-\ln L$ units better than the tree for which ratite monophyly is enforced, which in a pairwise comparison is rejected by KH and SH tests at $P = 0.136$.

With our own data set, both ingroup (Palaeognathae) and outgroup (Neognathae and Crocodilia) taxon sampling have been increased; RY-coding for protein third codon positions introduced; and substitution models are partitioned across protein codon positions and RNA stems and loops. Each of these changes is expected to reduce the influence of biases on phylogeny reconstruction. Our BI and ML bootstrap results reveal 2 differences from the mt consensus. The first is that the tinamou–moa grouping that was found in our reanalysis of the data set of Cooper et al. (2001) is now very strongly supported (99% for ML and 1.00 for BI, see Fig. 2). The second difference involves the placement of the ostrich. In Figure 1, the ostrich is shown to group with emu, cassowaries, and kiwi, regardless of ratite monophyly being enforced. In our new analyses, Figure 2 shows the ostrich diverging from the basal node among palaeognaths.

Higher substitution rates among the tinamous (see Fig. 3) than among the ratites should in theory be well accounted for by the partitioned rates-across-sites models. However, in order to check for consistency, analyses

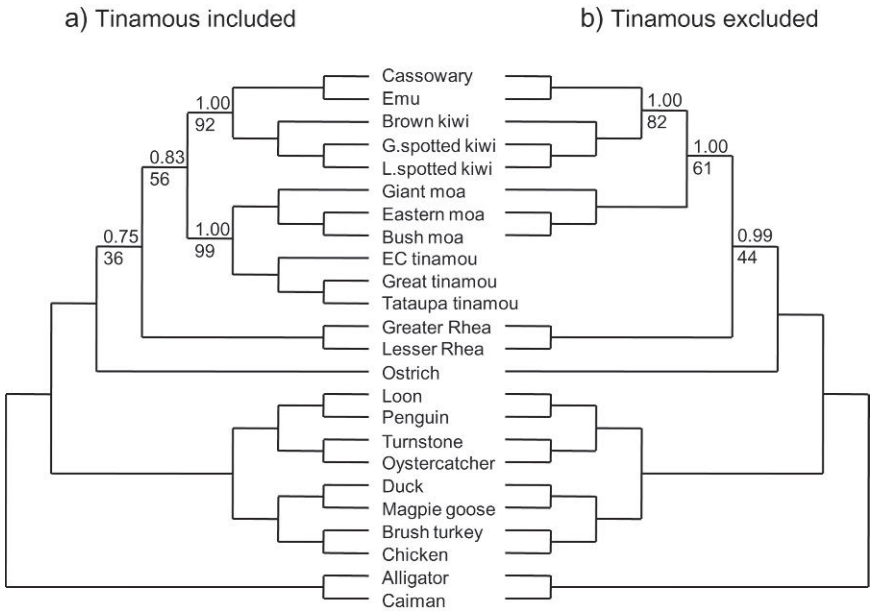

FIGURE 2. Avian phylogenies inferred from the complete mt protein- (third codons RY-coded) and RNA-coding DNA sequences (a) with and (b) without the inclusion of the tinamous. Support values are shown for Bayesian posterior probability (top) and ML nonparametric bootstrap (bottom). Values are not shown for nodes that receive maximum support in both analyses.

were also run without tinamous and it is encouraging that the favored tree (Fig. 2b) is congruent with the tree that includes tinamous (Fig. 2a). In fact, the ML bootstrap and BI support for all other palaeognaths grouping to the exclusion of the ostrich strengthens from 36% to 44% and 0.75 to 0.99, respectively.

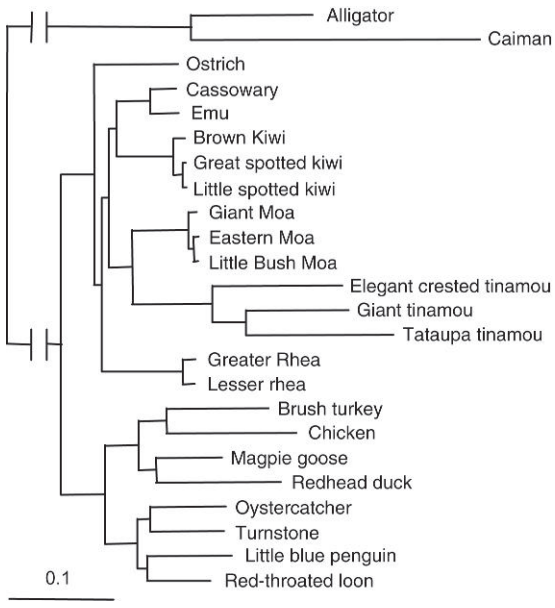

FIGURE 3. BI phylogram from the complete mt protein- (third codons RY-coded) and RNA-coding DNA sequences. The substitution model is partitioned across protein codons and RNA stems and loops, whereas rate multipliers were employed for branch-length estimation. Scale is substitutions per site.

ML Hypothesis Testing

Phylogenetic hypothesis testing using ML with partitions modeled separately circumvents both the concatenation problem associated with the ML bootstrapping in PAUP* and the restriction to parametric error estimation in MrBayes. Shimodaira and Hasegawa (1999) showed that such AU and KH hypothesis tests can more closely reflect sampling error than do bootstrap values, which in turn are far more faithful than typically overconfident Bayesian posterior probability (BPP) values (Suzuki et al. 2002; Gontcharov et al. 2004).

Among tinamous, moa, ostrich, rheas, kiwi, and Casuariidae, there are 945 possible rooted topologies. The combined ML scores for the separately modeled partitions and associated AU (and KH) P values are shown in Table 2(a) for the best 5 trees and several other trees of interest. The favored tree is the same as was found in the Bayesian and ML bootstrap analyses (Fig. 2) and groups moa with tinamous. The best tree containing the traditional split between tinamous and ratites (Tree 6) is consistent with Haddrath and Baker (2001), however, is over 37 $-\ln L$ units adrift from the overall best tree and is rejected at $P < 0.05$. Additionally, the Cooper et al. (2001) tree (7) is rejected at $P < 0.01$, as is a sister grouping of the 2 New Zealand taxa, moa and kiwi (Tree 8).

Beyond the sister groupings of tinamous and moa and of emus and cassowaries (Casuariidae), the relationships of the palaeognaths remain difficult to resolve. Although Table 2(a) shows the ostrich to be favored as the "basal-most" member, the second through fourth best trees include alternative basal-most taxa, tinamou–moa (2), rheas (3), and ostrich–rheas (4). None

TABLE 2. Log-likelihood differences between trees and their statistical significance under AU and KH tests (a) with tinamous included and (b) without tinamous

Alternative groupings[a]	$-\ln L$[b]	*P* values	
		AU	KH
a) Among Palaeognathae[c]			
1. (Out, (Ost, (Rhe, ((Moa, Tin), (Cass, Kiwi)))))	< 90, 905.8 >	—	—
2. (Out, ((Moa, Tin), (Rhe, (Ost, (Cass, Kiwi)))))	+1.9	0.597	0.448
3. (Out, (Rhe, (Ost, ((Moa, Tin), (Cass, Kiwi)))))	+4.2	0.462	0.307
4. (Out, ((Rhe, Ost), ((Moa, Tin), (Cass, Kiwi))))	+7.5	0.177	0.175
5. (Out, (Ost, (Rhe, (Cass, ((Moa, Tin), Kiwi)))))	+7.9	0.237	0.167
6. (Out, (Tin, (Moa, (Rhe, (Ost, (Cass, Kiwi))))))	+37.1	0.011*	0.047*
7. (Out, (Tin, (Rhe, (Moa, (Ost, (Cass, Kiwi))))))	+56.0	0.004*	0.008*
8. (Out, (Ost, (Rhe, (Cass, (Tin, (Moa, Kiwi))))))	+67.8	<0.001*	<0.001*
b) Among ratites only—tinamous excluded			
1. (Out,(Ost, (Rhe, (Moa, (Cass, Kiwi)))))	< 78, 982.2 >	—	—
2. (Out, (Ost, (Rhe, (Cass, (Moa, Kiwi)))))	+5.8	0.345	0.208
3. (Out, (Ost, (Rhe, (Kiwi, (Moa, Cass)))))	+7.0	0.231	0.158
4. (Out, (Rhe, (Ost, (Moa, (Cass, Kiwi)))))	+7.4	0.369	0.236
5. (Out, (Moa, (Rhe, (Ost, (Cass, Kiwi)))))	+10.4	0.365	0.282

Note: *significant at $P \leq 0.05$.

[a]The best 5 trees among the 945 and 105 possible trees for (a) and (b) are shown, respectively. Additionally for (a), the best tree in which ratites are monophyletic is Tree 6, which is congruent with Haddrath and Baker (2001). Tree 7 is congruent with Cooper et al. (2001), and in Tree 8 the New Zealand ratites are monophyletic.

[b]ML models are partitioned for proteins (by codon) and for RNA (stems, loops).

[c]Taxon abbreviations: Out, outgroup neognaths and crocodilians; Ost, ostrich; Rhe, rheas; Moa, moa; Tin, tinamous; Cass, Casuariidae.

of these alternative trees can be rejected at $P < 0.175$ for either the KH or the AU tests. Indeed, even a tree (5) in which tinamou–moa group with kiwi is rejected only at $P = 0.167$ (KH) and $P = 0.237$ (AU). In Table 2(b), the best 5 trees are shown for equivalent analyses but with the tinamous excluded. The results are consistent with those in Table 2(a) such that the ostrich diverging first among the ratites is not an artifact of the inclusion of the tinamous. Furthermore, these 5 trees in Table 2(b) are sufficient to show that each grouping within the favored ratite tree (ostrich, (rheas, (moa, (Casuariidae, kiwi)))) has an alternative that cannot be rejected at $P \leqslant 0.15$ for the KH test or at $P \leqslant 0.20$ for the AU test.

Cooper et al. (2001) sequenced a portion of the elephant bird (*Mullerornis agilis*) mt genome and found its affinities to lie with kiwi and Casuariidae effectively as an unresolved trichotomy. We examined whether the novel phylogenetic context (tinamou–moa and ostrich basal among palaeognaths) and RY-coded protein third codon positions would affect the placement of elephant birds. The available elephant bird sequences align against only 880 sites from our primary data set. These sites were analyzed alone and under a single GTR+I+Γ model in accord with concerns for overparameterization. ML scores were calculated in PAUP* for each possible placement for the elephant bird on the palaeognath tree that is shown in Figure 2a. The best placement was as sister to kiwi ($-\ln L = 6007.35$). Alternative placements as sister to either Casuariidae, kiwi + Casuariidae, tinamous + moa, or kiwi + Casuariidae + tinamous + moa could not be rejected at $P \leqslant 0.25$. Only elephant bird placements with the ostrich or as sister to all other palaeognaths could be rejected at $P \leqslant 0.10$.

Exploration for Nonphylogenetic Biases

Without RY-coding the protein third positions, BF differences divide ratites into 2 groups, kiwi, emu, cassowary, and ostrich with low-cytosine/thymine relative frequencies (average 1.59) and rheas and moa with high-cytosine/thymine relative frequencies (average 2.79). The non-palaeognath archosaurs also have high-cytosine/thymine relative frequencies (average 3.15) and so for the BF distance trees, kiwi, emu, cassowary, and ostrich group together strongly (Fig. 4). The tinamous have relatively low-cytosine/thymine relative frequencies (average 1.79). With their inclusion, the same relationships among ratites are recovered as in Figure 4, although tinamous are placed as sister to kiwi.

With our primary (RY-coded) data set, BF distances favor tinamous and rheas grouping on one side of the palaeognath root, whereas on the other side ostrich groups with Casuariidae and kiwi, then moa diverge progressively deeper from these. The groupings recovered in these optimal BF distance trees are only phylogenetically relevant when compared between tree topologies that are potential candidates for representing evolutionary history. Accordingly, Table 3 shows the ME differences (based on BF distances) between the alternative tree candidates from the partitioned ML analyses in Table 2(a). These comparisons reveal that similarity in base composition clearly favors ratite monophyly (Trees 6 and 7) for both the standard and the RY-coded nucleotide data. Importantly though, the RY-coding greatly reduces the potential for compositional heterogeneity to bias phylogenetic reconstruction. For example, the optimal tree from our phylogenetic analyses (Tree 1) is disadvantaged in terms of compositional heterogeneity relative to the favored BF distance tree (Tree 7) by 128.4 changes before RY-coding but only by 24.4 changes after RY-coding. As such, these results are consistent with compositional heterogeneity contributing toward "apparent" phylogenetic signal for ratite monophyly and more so in studies that have not applied RY-coding to protein third positions. Notably, the favored BF distance trees 7 and 6 are consistent with the favored trees from Cooper et al. (2001) and Haddrath and Baker (2001), respectively.

High rates of substitution among the tinamous and outgroup taxa relative to the ratites should provide

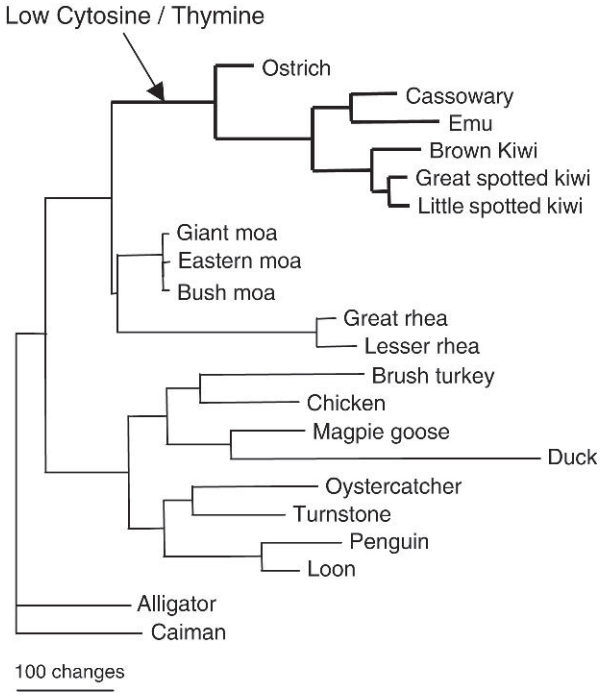

FIGURE 4. Minimum evolution tree on BF distances from the complete mt protein- and RNA-coding DNA sequences. Compositional bias favors ostrich grouping with kiwi and Casuariidae. Average cytosine/thymine (C/T) among these taxa is 1.59. Average C/T among the moa and rheas is 2.79, close to the average among the outgroup taxa (3.15).

artifactual "long-branch-attraction" signal for ratite monophyly if hidden substitutions are undercorrected for. This is indeed the case; for both the standard and the RY-coded data sets, minimum evolution on uncorrected

TABLE 3. BF distance minimum evolution differences between the tree topologies that were compared in Table 2(a) for ML scores

Alternative palaeognath trees[a]	a. Ptn123rna (all nucleotide)[b]	b. Ptn123rna (3RY)[c]
1. (Out, (Ost, (Rhe, ((Moa, Tin), (Cass, Kiwi)))))	+128.4	+24.4
2. (Out, ((Moa, Tin), (Rhe, (Ost, (Cass, Kiwi)))))	+85.7	+21.7
3. (Out, (Rhe, (Ost, ((Moa, Tin), (Cass, Kiwi)))))	+93.1	+3.0
4. (Out, ((Rhe, Ost), ((Moa, Tin), (Cass, Kiwi))))	+122.9	+66.8
5. (Out, (Ost, (Rhe, (Cass, ((Moa, Tin), Kiwi)))))	+174.0	+36.7
6. (Out, (Tin, (Moa, (Rhe, (Ost, (Cass, Kiwi))))))	+65.3	+2.0
7. (Out, (Tin, (Rhe, (Moa, (Ost, (Cass, Kiwi))))))	< 3008.3 >	< 1175.9 >
8. (Out, (Ost, (Rhe, (Cass, (Tin, (Moa, Kiwi))))))	+207.8	+29.1

[a]The first 5 trees are the best ML trees. Trees 6 and 7 are the best supported by BF distances and are consistent with ratite monophyly. Tree 8 groups the New Zealand ratites (moa and kiwi). See Table 2 for taxon abbreviations.
[b]Standard nucleotide coding.
[c]Protein third codon positions RY-coded.

TABLE 4. The influence of the gamma shape parameter (α) on ML support for the placement of tinamous either as in the favored (ratite paraphyly) tree in Table 2 (Tree 1) or as in the best tree with ratite monophyly constrained (Tree 6)

Gamma shape parameter (α)	ln$L$ units favoring ratite paraphyly over monophyly[a]	KH P value[b]
Infinity	32.1	<0.001
2.000	37.1	<0.001
1.000	38.4	<0.001
0.750	38.7	<0.001
0.500	39.1	<0.001
0.358[c]	40.0	<0.001
0.300	41.0	<0.001
0.250	42.6	<0.001
0.200	44.5	<0.001

[a]The ML model used is identical to that used for the ML bootstrap analyses, except that α is varied.
[b]For pairwise comparisons, the KH test is equivalent to the SH test.
[c]The ML value for α was 0.358.

(p) distances drew tinamous toward the outgroup, leaving ratite monophyly (not shown). Similarly, rate heterogeneity that is unaccounted for in our models for the ML and Bayesian analyses might lead to the present support for a tinamou–moa grouping in fact being underestimated relative to ratite monophyly. Conversely, if our I+Γ models overestimate hidden substitutions (and gamma models do not always provide a good fit, see Susko et al. 2003), then long-branch repulsion (see Siddall 1998) may be providing artifactual signal for grouping the tinamous among the far-slower evolving ratites.

The ML model optimized for the bootstrap analysis on the concatenated data (with 3RY) had a gamma shape parameter of 0.358. Under this model, the tinamou–moa grouping is favored over ratite monophyly by 40.0 $-$ln$L$ units. By varying only the gamma shape parameter, we were able to get an appreciation for the influence of lesser or greater correction for hidden substitutions, which should encourage long-branch attraction and repulsion, respectively. Hence, Table 4 shows that as expected, the support in favor of tinamous grouping with moa increases upon lowering the shape parameter and decreases upon increasing the shape parameter. The results suggest that it is possible that our primary analyses slightly underestimate or overestimate the support for tinamou–moa. Importantly though, even with the shape parameter (miss)specified at infinity, ratite monophyly is still rejected at $P < 0.001$.

Molecular Dating and Ancestral State Reconstruction

The median node heights from the BEAST molecular dating analysis provide the scale for the chronogram in Figure 5. Note that only the divergence of the ostrich at the basal palaeognath node occurs before the break-up of South Gondwana involved landmass separation with New Zealand rifting from Antarctica and starting to "unzip" from Australia. In terms of the terrestrial dispersal history of modern faunas, this division of South Gondwana is essentially complete with the isolation of

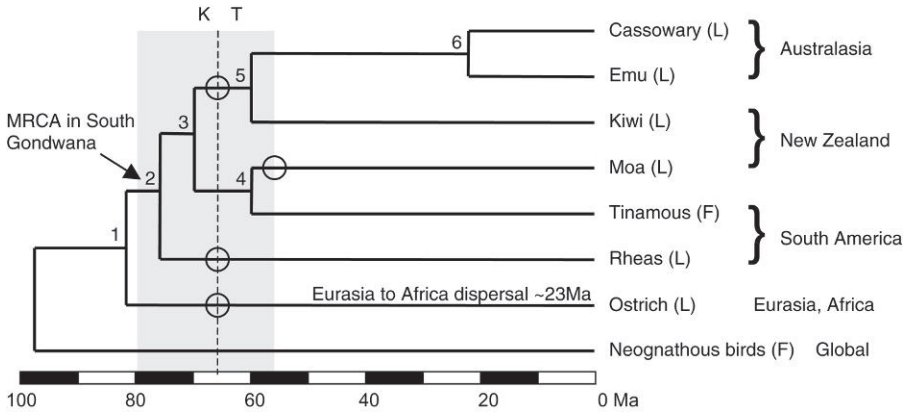

FIGURE 5. Timescale of palaeognath evolution with relative placements indicated for inferred losses of flight (circles), the Cretaceous-Tertiary boundary (dotted line), and the major period of fragmentation of South Gondwana (shaded). Inferred median dates from the BEAST analysis of the 24-taxon tree for palaeognath divergences at the numbered nodes in millions of years before present: (1) 83.4, (2) 76.4, (3) 70.6, (4) 60.0, (5) 59.9, (6) 20.7. 95% HPDs are shown in Table 5. Elephant birds of Madagascar have not been included due to the uncertainty of their phylogenetic placement. F and L in parentheses after taxon names denote flighted and flightless, respectively.

Australia and South America by about 55 Ma (Woodburne and Case 1996). Median estimates and 95% HPDs for palaeognath divergences are shown in Table 5(a). The dates noted below and which were subsequently used for the ancestral character state and dispersal analyses employed only the 5 calibration ranges that are external to Palaeognathae. The results with the additional inclusion of 2 preliminary internal calibrations (emu vs. cassowary and rhea vs. kiwi/Casuariidae/tinamou–moa) are also shown in Table 5(b).

After the Ostrich diverges at 83 Ma, the origin of the South Gondwanan clade of palaeognaths is dated at 76 Ma. Two of the deep divergences among palaeognaths, kiwi from Casuraiidae and tinamous from moa, are estimated to be post-K/T, at 60 Ma. The analyses with the internal palaeognath calibrations provide slightly younger dates still, which encouragingly, have far narrower 95% HPDs.

Dispersal analyses were conducted for both the overall rooted 24-taxon tree (O_{24}) tip-labeled as Northern Hemisphere, Africa, and South Gondwana and the rooted 13-taxon South Gondwanan clade (SG_{13}) tip-labeled as South America, Australasia, and New Zealand. In order to examine how robust the results were to incorporating information from the fossil record, 3 key fossil palaeognaths were included. These were the 42 Ma European *Paleotis* as sister to ostrich (Houde 1986), the ≈56 Ma Brazilian *Diogenornis* as sister to Rhea (Alvarenga 1983), and the ≈70 Ma oldest known lithornithid (Parris and Hope 2002) from North America. Sampling in the fossil record is too sparse to accurately infer how long it was because each of these fossils diverged from their sister taxa. For the chronogram used in the BayesTraits analyses, we nominally placed the divergences of these taxa from their sister taxon 10 Ma prior to the fossil age (of *Paleotis* and *Diogenornis*) or 10 Ma prior to the crown palaeognath root (for the lithornithid). Such timing allows the fossil taxa to inform the ML analyses without constraining their common ancestor with the modern taxa to share the same state. With the fossil taxa included, the overall and South Gondwanan dispersal analyses are referred to as O_{27} and SG_{14}. Note that among the fossil taxa only *Diogenornis* falls within the SG clade.

TABLE 5. Palaeognath divergence estimates in Ma from BEAST analyses

Node[c]	(a) external calibrations[a]		(b) external and internal calibrations[b]	
	Median	95% HPD	Median	95% HPD
1	83.4	55.2–103.6	78.1	60.4–94.0
2	76.4	50.3–97.8	70.0	57.0–82.4
3	70.6	46.6–92.5	63.8	50.4–79.1
4	60.0	38.3–81.6	53.2	39.1–71.5
5	59.9	36.5–82.7	53.5	36.9–72.1
6	20.7	7.6–50.9	28.6	25.0–34.3

[a]With only 5 calibration ranges for nodes external to palaeognaths.
[b]With the addition of 2 calibration ranges within Palaeognathae.
[c]Node numbers refer to Figure 5.

The results of the ML dispersal analyses are shown in Table 6. In both the O_{24} and O_{27} analyses, a South Gondwanan origin is preferred for the MRCA of modern palaeognaths. However, with the fossil taxa included the probability of a Northern Hemisphere origin is 0.18 and in fact becomes favored under MP (not shown). South Gondwanan origins are very strongly supported for the non-ostrich palaeognaths (Node 2 in Table 6) in all analyses, and more specifically favored to be in South America, particularly for SG_{14}, although New Zealand origins cannot be rejected at $P \leqslant 0.05$. None of the analyses (including MP) clearly distinguish between South American origins for kiwi/Casuariidae/tinamou–moa (Node 3 in Table 6) with independent dispersals of kiwi and moa to New Zealand or, alternatively, an earlier dispersal of Node 3 ancestors from

TABLE 6. ML inference of palaeognath dispersal history

Node[c]	Overall tree O_{27} (O_{24})[a]			South Gondwana clade SG_{14} (SG_{13})[b]		
	Northern Hemisphere	Africa	South Gondwana	South America	Australia	New Zealand
1	0.18 (0.00)	0.00 (0.09)	0.82 (0.90)	—	—	—
2	0.00 (0.00)	0.00 (0.00)	1.00 (0.99)	0.90 (0.55)	0.01 (0.03)	0.09 (0.42)
3	—	—	—	0.34 (0.28)	0.09 (0.09)	0.55 (0.62)
4	—	—	—	0.67 (0.69)	0.02 (0.01)	0.31 (0.30)
5	—	—	—	0.09 (0.05)	0.52 (0.55)	0.38 (0.39)

[a]Probabilities for the overall tree analyses (O_{24}, O_{27}) are provided for MRCAs at Nodes 1 and 2 being in the Northern Hemisphere, Africa, or South Gondwana. In each case, the probabilities are given both from analyses in which information from fossil taxa was included (and excluded).

[b]South Gondwana is divided into South America, Australasia, and New Zealand only for Nodes 2–5 (for SG_{13}, SG_{14}). These analyses are nested within the overall tree in recognition of dispersal probabilities being nonhomogenous across the tree and that dividing South Gondwana is only meaningful after approximately 80 Ma.

[c]Node numbers refer to Figure 5.

South America to New Zealand (or Australasia) and a later return for tinamou ancestors to South America. Support for the origination of the kiwi/Casuariidae clade (Node 5) in either Australasia or New Zealand is similarly difficult to tease apart.

Without obvious a priori reasons to expect the fragmentation of Southern Gondwana to influence the evolution of flight/flightlessness, we return to single analyses with the O_{24} data set. Loss of flight in the ancestors of palaeognaths and regain in the ancestors of tinamous are favored under standard MP. Ancestral character state reconstruction for O_{24} under ML is similar, except that slightly earlier regain of flight in the ancestors of tinamou–moa is favored at $P = 0.78$ (Table 7c). Lithornithids were flying birds (Houde and Olson 1981), whereas the flight status of *Paleotis* and *Diogenornis* is uncertain. Incorporating lithornithids and fixing the archosaur and avian roots to be flightless and flying, respectively (in agreement with essentially all fossil evidence and phylogenetic inference), make little difference to the ML character state reconstruction (Table 7d). A further modification involved artificially biasing the ML analyses in favor of flight loss over gain by assuming that we had instead sampled flightless duck and megapode species. As shown in Table 7, the ML estimates for the expected ratio of loss to gain of flight increases substantially and flightlessness cannot be rejected even at $P \leqslant 0.20$ at any of the palaeognath nodes 1–4. Dollo Parsimony in which flight cannot be regained once it is lost favors flight being lost independently in ostrich, rheas, moa, and kiwi/Casuariidae.

DISCUSSION

Palaeognath Phylogeny

Monophyly of ratites and their sister relationship with tinamous are two of the best agreed upon hypotheses of avian supraordinal phylogeny that derive originally from morphological data (e.g., Bock 1963; Cracraft 1974) and then from nuclear data (Prager et al. 1976; Sibley and Ahlquist 1990; van Tuinen et al. 2000). Analyses of complete mt genome sequences (Cooper et al. 2001; Haddrath and Baker 2001) appeared to offer substantive confirmation of both, as well as a "mt consensus" on relationships among palaeognaths that has been widely adopted (Fig. 1a). However, one of two fundamental problems exist for all previous phylogenetic analyses of palaeognath mt genomes for which tinamous, moa, and at least one other ratite were included. Either rates-across-sites heterogeneity was not accounted for, or when it was (e.g., under a gamma distribution) ratite monophyly was enforced. Upon relaxing this phylogenetic constraint with the data set of Cooper et al. (2001), tinamous and moa group together, rendering ratites paraphyletic.

Our primary analyses incorporate 5 functionally distinct partitions (protein codons 1, 2, 3 and RNA stems and loops) and RY-coded third positions. These retain

TABLE 7. Inference of the evolutionary history of flight (F) and flightlessness (L) among palaeognaths and the probability of being flighted

Node[b]	Ancestral state reconstruction models[a]				
	a. MP	b. MP-Dollo	c. ML (mod)	d. ML (lith)	e. ML (lith/alt)
1. Palaeognathae	L	F	0.02	0.04	0.24
2. Node 2	L	F	0.05	0.08	0.31
3. Node 3	L	F	0.17	0.22	0.46
4. Tinamou–moa	L	F	0.78	0.79	0.81
5. Kiwi/Casuariidae	L	L	0.02	0.04	0.13
Expected P(loss)/P(gain)	1.00	1/0	1.46	1.58	2.34

[a](a) Standard and (b) Dollo MP and the probability of being flighted according to ML analyses in which (c) only modern taxa were included, (d) a lithornithid was added, and in addition (e) a flightless duck and megapode were artificially sampled.

[b]Node numbers refer to Figure 5, such that Node 2 includes all modern palaeognaths except ostrich and Node 3 includes kiwi/Casuariidae/tinamou–moa.

one aspect of the mt consensus with solid, though not irrefutable support, emu and cassowary (Casuariidae) grouping with kiwi. Being able to confidently reject the closest alternative (Table 2a, Tree 4), in which tinamou–moa group with kiwi, will require further examination. Nuclear data may be important here. Harshman et al. (2008) were also unable to confidently resolve the relative affinities of kiwi, rheas, tinamous, and Casuariidae, although encouragingly their analyses favored the same topology we present for mt data (see Fig. 2).

Determining the earliest divergence among the ratites also requires further study. However, our analyses are consistent with the ostrich diverging from the MRCA node of ratites (and indeed palaeognaths). Support for this hypothesis increases further (from 1.9 to 7.4 ln$L$ units) with the exclusion of the tinamou sequences from the analyses (cf. Table 2a,b). The conflicting signal that supports the alternative rooting of ratites in the mt consensus, which places rheas and moa deepest, appears to derive largely from compositional bias. This is evidenced by rhea and tinamou–moa falling to the base of Palaeognathae if RY-coding is not used to reduce compositional bias at third positions in our Bayesian analyses (under both DNA and codon models, not shown).

The BF distance tree in Figure 4 shows that a long branch groups the ostrich with Casuariidae and kiwi. As noted in that figure, these ratite mt genomes have low cytosine relative to thymine when compared with the rheas, moa, and outgroup taxa. With the inclusion of the tinamous and compared among the best topologies from the partitioned ML analyses, the topologies that are best supported by standard (nucleotide) BF distances (Table 3a, Trees 6 and 7) are in fact the topologies supported by Haddrath and Baker (2001) and Cooper et al. (2001). These 2 trees remain the best supported by BF distances on the RY-coded data (Table 3b), although the recoding greatly reduces the magnitude of this artifactual support for placing tinamous outside of ratites and rheas/moa deepest among ratites.

Haddrath and Baker (2001) recognized the problem of compositional heterogeneity for inferring palaeognath phylogeny. However, the potential solutions they employed (LogDet distances [Lockhart et al. 1994] and nonhomogenous ML, NHML [Galtier and Gouy 1998]) did not allow for RAS variation. As noted earlier, accounting for RAS variation is critical for reducing branch-length artifacts. Moreover, ignoring RAS variation leads to the influence of compositional nonstationarity being underestimated (see Phillips et al. 2004). More recent versions of NHML incorporate RAS, although still assume that compositional heterogeneity conforms to the AT/GC pattern that is common for nuclear DNA and that among the BFs, A = T and G = C. Instead, the ML optimized "equilibrium" frequencies among the 2 purines and 2 pyrimidines differ considerably and compositional heterogeneity among the avian mt sequences primarily concerns variation among pyrimidines (C,T) specifically.

Our analyses favor the ostrich diverging from the base of Palaeognathae, in line with some previous mt studies that did not include moa (e.g., van Tuinen et al. 1998; Harrison et al. 2004). Importantly, compositional nonstationarity provides a "smoking gun" for explaining the deeper placement of moa and rheas in most previous studies. Hence, on both mt and nuclear data (e.g., Harshman et al. 2008) an early ostrich divergence must now be considered the best phylogenetic estimate on which to base evolutionary and biogeographical inferences. Another result, the tinamou–moa grouping is more salient; all ML bootstrap and BPP support values are $\geqslant$99% (Fig. 2) and partitioned ML hypothesis testing rejects the best tree with ratite monophyly (Table 2a, Tree 6) at $P < 0.05$.

It is encouraging that with the tinamou sequences excluded, moa maintain a concordant placement in the tree, as sister to Casuariidae and kiwi. Curiously, with moa excluded, tinamous tend to fall to a basal placement among palaeognaths (not shown, but as for Harrison et al. 2004). This situation is reminiscent of earlier efforts to root the tree of placental mammals (e.g., Kretteck et al. 1995). Analogous to tinamous, hedgehog sequences evolved at very high rates and were artifactually attracted to the base of Placentalia. It was not until the hedgehogs could be bound to a sufficiently close (and slower evolving relative), a shrew, that hedgehog affinities were shown to be nested well within Placentalia (Lin et al. 2002). One might speculate that for both the hedgehogs and the tinamous, the branch-length artifacts are partly associated with heterotachy, for which standard stationary models can be inconsistent (see Lockhart et al. 1998). If this is the case, then the importance of the shorter branch-length moa and shrews for binding their longer branch-length cousins while retaining deeper signals of ancestry is not surprising.

The first studies to include complete mt sequences from tinamous, moa, and other ratites in 2001 could have recovered the tinamou–moa grouping (see Fig. 1b). However, either ratite monophyly was enforced or RAS ML results were overlooked. It may be significant that in the lead-up to these papers several highly anomalous phylogenetic relationships were proposed on the basis of mt genome analyses, including passerines being sister to all other extant birds (e.g., Mindell et al. 1999) and the egg-laying monotremes grouping with marsupials among mammals (Janke et al. 1996). Ancient DNA studies were also in their infancy and were viewed with some suspicion (see Cooper and Poinar 2000). Indeed, we too were guilty of these mistrusts, having left moa out of an avian mt genome phylogeny (Harrison et al. 2004) partly because their inclusion induced the "wrong" tree, in which tinamous fell inside ratites.

Additional taxon sampling and advances in analytical methods have since resolved earlier anomalies for mt genome phylogenies, including for rooting the avian and mammalian trees (see Paton et al. 2002; Phillips and Penny 2003). Furthermore, ancient DNA is now a respectable and thriving industry. Having ruled out compositional and long-branch artifacts (Tables 3 and 4), we consider the present mt genome analyses to provide

strong and unambiguous support for tinamous and moa being sister taxa and hence ratites being paraphyletic.

Independent Evolution of Flightless Palaeognaths

Multiple losses of flight among ratites have often been suggested, typically under the influence of a need to explain apparent requirements for long-distance dispersal over water (e.g., Houde 1988; Briggs 2003). Harshman et al. (2008) suggested at least 3 losses of flight among palaeognaths, as inferred from ratite paraphyly in their phylogenetic tree and a belief that flight is more likely lost than regained among birds. Alternatively, they recognized that a flightless MRCA of palaeognaths and subsequent regain of flight in tinamous was the most parsimonious option. Standard MP is essentially no-common-mechanism ML (Tuffley and Steel 1997), and so reconstruction at any given node is not informed by patterns at other nodes that indicate that loss of flight is more likely than gain.

In Table 7(c–e), it is shown that as the expected ratio of flight loss to gain increases from 1.46 to 2.34 through various ML ancestral state reconstruction analyses, the probability of each of the palaeognath MRCAs being flighted (and hence, of subsequent flight losses) increases. Indeed, 4 losses of flight among palaeognaths cannot be rejected even at $P \leqslant 0.2$ in the latter scenario that assumes we had sampled 2 flightless galloanserae. Even here a flight loss/gain ratio of 2.34 is far too conservative given hundreds of reported flight losses among birds (as discussed below) and no clear evidence for any regains. Hence, our prior expectation approaches the Dollo parsimony situation with regains not permitted, such that losses are required along each of the ostrich, rhea, moa, and kiwi/Casuariidae stem lineages (as shown in Fig. 5). If the MRCA of Casuariidae and kiwi (for which our best estimate of 60 Ma postdates New Zealand–Australia separation) was volant and so was their MRCA with elephant birds, then up to 6 losses of flight among palaeognaths are required.

Loss of flight has occurred very frequently among birds; McCall et al. (1998) suggested loss of flight occurred in at least 11 extant avian families and Steadman (2006) reported more than 100 losses of flight on the Pacific Islands among the Rallidae alone. Indeed, Maynard-Smith (1968), Feduccia (1996), Bautista et al. (2001), and others have argued that theory predicts that flight will tend to be lost in the absence of direct selection for its maintenance (catching food on the wing, predator avoidance, etc.). Wings and associated pectoral apparatus are costly to maintain, and walking is more economical for ground-feeding birds if food sources are not widely distributed. Moreover, the "cost" of powered flight increases with the linear dimension (l) in proportion to $l^{3.5}$, whereas the power from the muscle (or the area of the wing) only increases in proportion to l^2 (Maynard-Smith 1968, pp. 13–14). Hence, loss of flight is not surprising given that larger size has clearly been selected for among ratites, relative to the typically chicken-sized, volant and probably para- or polyphyletic assemblage of lithornithids they are thought to derive from (see Leonard et al. 2005). By contrast, regaining flight in tinamous after at least 20 Ma of selection for flightless locomotion and erosion of previously evolved genetic architecture associated with all aspects of flight is implausible, especially in the presence of already flight-adapted competitors.

Morphological support for ratite monophyly to the exclusion of tinamous is typically strong in cladistic analyses (e.g., Livezey and Zusi 2007). However, many of the ratite synapomorphies are directly associated with flightlessness or have previously been suggested in various groups to be developmentally correlated through paedomorphosis (Livezey 1995; Härlid and Arnason 1999). Interestingly, Elzanowski (1995) focused his morphological study primarily on the basicranuim, which he believed to have relatively little functional/developmental association with flightlessness and as a result found tinamous falling within ratites and the ostrich deepest among palaeognaths. Even so, Cubo and Arthur (2001) showed that numerous cranial (not basicranial) characteristics appear to be developmentally correlated with the pelvic peramorphosis that occurs in flightless birds, particularly the more cursorial taxa. A number of authors (e.g., Jollie 1977; Härlid and Arnason 1999) have proposed such heterochronous developmental mechanisms to have been of fundamental importance for understanding the evolution of ratite morphologies. Additionally, differential character scaling with size (allometry) also disguises developmental correlations that can pose as phylogenetic signal (Szalay 1994).

If character correlations associated with allometric scaling and cursoriality contribute substantially to morphological signal for ratite monophyly, then it is predictable that kiwi, the smallest and least cursorial ratite, might be attracted toward a basal position in morphological cladistic analyses. Indeed, this has most often been the case. Livezey and Zusi (2007) grouped the more cursorial ratites to the exclusion of kiwi with 92% MP bootstrap support, whereas Houde (1986) advocated kiwi arising independently of other ratites, from a different lineage among lithornithids. Hopefully, the present molecular results will encourage some reconsideration of avian morphological character analysis. Utilizing the tinamou–moa grouping within a molecular phylogenetic scaffold for morphological studies may benefit inferences of character state polarities.

One of the curiosities of flight being lost independently among several ratite lineages is the implication that flying lithornithid-like lineages likely became extinct independently in North America, Eurasia, Madagascar, New Zealand, and Australia–Antarctica, with only the tinamous surviving in South America. All palaeognaths appear to have become extinct during the Tertiary in the northern continents, until the later rearrival of ostriches. The avian fossil records of the abovementioned Gondwanan landmasses are very poor or entirely missing from the Late Cretaceous through to

the Early Tertiary and contain no records of lithornithid-like birds. However, we might imagine scenarios similar to the more recent and transparent case of swamphens (*Porphyrio sp*.) flying to New Zealand, where ecological conditions differed from those for their ancestral population (an absence of mammalian predators in this case). Apparently selection for larger, flightless birds simultaneously resulted in the evolution of the Takahe (*Porphyrio hochstetteri*) and the elimination of flying swamphens—until humans and their commensals arrived (Trewick 1996).

Vicariance biogeography provides the most familiar hypothesis for the diversification of ratites as ancestrally flightless cousins set adrift upon the break-up of Gondwana (Cracraft 1974). Much of the Gondwanan break-up occurred too early for this to be plausible (see below). Here we suggest a new hypothesis that flying palaeognaths accessed similar novel niche opportunities that became available on different landmasses with the K/T boundary extinction of dinosaurs and in the absence of previously overwhelming predation pressures, independently became flightless. Consistent with this proposal, Figure 5 shows that each of the branches along which flight was apparently lost either crosses or originates after the K/T boundary. Relevant fossil evidence is sparse, but nonetheless fits. Flying lithornithids are known from the Late Cretaceous (Parris and Hope 2002), whereas the earliest fossil records for putatively flightless palaeognaths, which were somewhat larger than lithornithids, are known from just a few million years after the K/T boundary in Palaeocene deposits from Argentina (Alvarenga 1993) and possibly France (Martin 1992). Perhaps, the apparent mimicry between ratites such as the ostrich (*Struthio*) and dinosaurs such as *Struthiomimus* is more closely linked than previously recognized.

Palaeognath Biogeography and the Question of Gondwanan Origins

The geographic distribution of extant palaeognaths and moa is shown in Figure 5. Our proposal of 4–6 losses of flight among the ancestors of recent palaeognaths invalidates a major underpinning of the Gondwanan vicariance model for explaining this distribution, the assumption that the MRCA of ratites was flightless. ML probabilities for the geographic location of MRCAs at the 5 deepest palaeognath nodes are shown in Table 6.

Interpretations based on vicariance and the mt consensus both place the last common ancestor of ratites and potentially palaeognaths in Gondwana. In contrast, we are unable to give preference to either a Northern Hemisphere origin with a common ancestor of non-ostrich palaeognaths dispersing to South America or a Gondwanan origin with an ostrich ancestor dispersing from South America to the Northern Hemisphere. With fossil information included in our analyses, ML favors a South Gondwanan origin, though does not clearly reject a Northern Hemisphere origin ($P = 0.18$), which is in fact favored under MP. Fundamental to both reconstructions is a late migration of ostriches to Africa. Most studies (e.g., Cooper et al. 2001; Slack et al. 2006), including ours, have placed the divergence of ostriches from an ancestor with other ratites that was too recent to catch the splitting of Africa from South Gondwana. A sister relationship for the ostrich with the NZ–Australian clade in results influenced by compositional biases fitted better with the possible use of (still uncertain) connections between Antarctica, the Kerguelen plateau, and Indo-Madagascar as a dispersal route to Africa. Instead, the basal placement of the ostrich favors a Late Cretaceous proto-Antilles dispersal route between South America and North America as has been suggested by van Tuinen et al. (1998). Notably, this temporal window for traversing the proto-Antilles land bridge also appears to have been used for the northerly migration of titanosaurid dinosaurs and vice versa for marsupials (see Pascual 2006).

Eurasian origins for ostriches have previously been proposed on the basis of both the possibility of separate origins of ratites from among lithornithids and the earliest fossil evidence for the lineage being from Eocene deposits in Europe (*Paleotis*, Houde 1986). Furthermore, the first ostriches (or indeed ratites) known from Africa are not revealed in the fossil record until the Miocene (Leonard et al. 2006), just subsequent to the first major Eurasian-African biotic interchange (Kappelman et al. 2003). In either case, the evidence does not hold up the status of ratites as the iconic Gondwanan taxon—with origins that predate the supercontinent's major break-up events and that later dispersed in accordance with vicariance and remnant ridges/microcontinents (of uncertain temporal and geographic continuity between the major landmasses). Furthermore, we confirm the view of Cooper et al. (2001) that elephant birds are not closely related to the ostrich. These Madagascan ratites appear to derive from among the Casuariidae/kiwi/tinamou–moa clade, with which they would share a South Gondwanan MRCA. The oceanic barrier between these lands and Indo-Madagascar had opened up by 110 Ma (Hay et al. 1999), such that either flight or extensive emergence of the Kerguelan Plateau would appear to necessitate dispersal.

A South Gondwanan origin for the MRCA of the "non-ostrich" palaeognaths is supported unequivocally by the nested ML dispersal analyses (Node 2, Fig. 5). More specifically, with the fossil information included for just this South Gondwanan clade, a South American origin is strongly supported ($P = 0.90$, Table 6, Node 2).

Our ML analyses do not clearly favor either of 2 scenarios for dispersal of palaeognaths out of South America into Antarctica–Australia–New Zealand (see Table 6, Nodes 3–4). These being a single dispersal, followed by a back migration to South America for tinamous or 2 dispersals, one for the ancestors of kiwi/Casuariidae and another for the ancestors of moa. We prefer the latter scenario because the presumed longer history of

palaeognaths in South America and that continent's present richness of tinamous hint at greater potential as a dispersal source. Admittedly, the paucity of Late Cretaceous/Early Tertiary fossil evidence from Antarctica–Australia–New Zealand ensures that this reasoning is tentative. Nevertheless, the best estimates from either of our molecular timescales for any of these dispersals out of South America are contained within 76 and 53 Ma (see Table 5, between Nodes 2 to 3 and 2 to 4/5). These match the dates for marsupials entering Australia via trans-Antarctic dispersal from South America between 72 and 55 Ma (Woodburne and Case 1996; Beck 2008). The finding by Tambussi et al. (1994) of ratite fossil material from Eocene deposits on Seymour Island, Antarctica is consistent with similar timing for ratite dispersal. Hence, palaeognath dispersals between Australia–Antarctica and between the southern and northern hemispheres (and ultimately Africa) may have been associated with major faunal migration intervals. In contrast, a plausible explanation for the origins of palaeognaths in New Zealand and Madagascar is provided by retention of flight well beyond the MRCA of the ratite birds.

Conclusions

Previous mt genome analyses of palaeognath birds have either constrained ratites and tinamous to be reciprocally monophyletic or have relied on analyses in which rate variation among variable sites is ignored. Relaxing these topological and methodological constraints leads to moa and tinamous grouping together. Increased taxon sampling and RY-coding the rapidly evolving third codon positions reduces tendencies for long-branch attraction between the tinamous and the outgroup taxa and provides strong statistical support for a tinamou–moa grouping. The implication that flight has been lost multiple times among palaeognaths calls into question the use of continental break-up dates for calibrating molecular clocks. In addition, the central role ratites have played in arguments concerning Gondwanan biogeography and the proposed Oligocene drowning of New Zealand (Waters and Craw 2006) need to be reevaluated.

Our phylogenetic reconstructions and examination of nucleotide composition bias suggest that ostriches diverged from the root node of Palaeognathae, in agreement with recent nuclear studies (Hackett et al. 2008; Harshman et al. 2008). Our biogeographic reconstructions are consistent with early palaeognath dispersal to or even origins in the northern continents. In turn, this helps explain the absence of African ratites until after the End Oligocene biotic interchange with Eurasia, which substantially postdates Eocene fossil records for apparent ostrich relatives in Eurasia.

Inferences from taxonomic/phylogenetic diversity alone have been unable to uncover remnant signal among modern terrestrial vertebrates for an evolutionary response to the end Cretaceous events (e.g., Bininda-Emonds et al. 2006). Multiple losses of flight coincident with size increases among palaeognaths independently on different landmasses following the end Cretaceous extinction event is suggestive of such a signature. We propose that large size and cursoriality and consequently loss of flight were selected for among Early Tertiary ancestors of modern ratites in filling parts of the ecospace vacated upon the K/T boundary extinction of mid–large-sized terrestrial vertebrates, including dinosaurs. Further resolution of palaeognath relationships and a more precise timescale will be crucial for testing this hypothesis and further unravelling the evolutionary history of flightless palaeognaths.

Supplementary Material

Supplementary material can be found at http://www.sysbio.oxfordjournals.org/.

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APPENDIX

Reanalysis of the mt Data Set from Cooper et al. (2001)

The data set of Cooper et al. (2001) includes 10,767 aligned mt protein-coding sites for the chicken and 9 palaeognaths, including 2 tinamous, rhea, ostrich, cassowary, emu, kiwi, and 2 moa. Note that the suffix -s is not added to kiwi and moa in their plural forms, in accordance with the Maori language from which they are derived. The copy of this data set that we obtained did not include the chicken sequence. We added the chicken mt sequence and in order to check that its aligned inclusion closely matched that used in the original analyses, we replicated the ML bootstrap analysis from Cooper et al. (2001). This analysis employed a single GTR+Γ model for the concatenated protein-coding DNA sequences, which was run in PAUP*4.0b10 for 200 nonparametric pseudoreplicates. With ratite monophyly enforced, the same topology was recovered as in the original paper and bootstrap support was similar. Differences in bootstrap support from the published analysis averaged only 2.3% across all nodes, which may largely be accounted for by stochastic variation in bootstrap sampling. When ratite monophyly was not enforced, tinamous and moa grouped together as shown in Figure 1b.