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Ratite Nonmonophyly: Independent Evidence from 40 Novel Loci

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Abstract.—Large-scale multilocus studies have become common in molecular phylogenetics, but the best way to interpret these studies when their results strongly conflict with prior information about phylogeny remains unclear. An example of such a conflict is provided by the ratites (the large flightless birds of southern land masses, including ostriches, emus, and rheas). Ratite monophyly is strongly supported by both morphological data and many earlier molecular studies and is used as a textbook example of vicariance biogeography. However, recent studies have indicated that ratites are not monophyletic; instead, the volant tinamous nest inside the ratites rather than forming their sister group within the avian superorder Palaeognathae. Large-scale studies can exhibit biases that reflect a number of factors, including limitations in the fit of the evolutionary models used for analyses and problems with sequence alignment, so the unexpected conclusion that ratites are not monophyletic needs to be rigorously evaluated. A rigorous approach to testing novel hypotheses generated by large-scale studies is to collect independent evidence (i.e., excluding the loci and/or traits used to generate the hypotheses). We used 40 nuclear loci not used in previous studies that investigated the relationship among ratites and tinamous. Our results strongly support the recent molecular studies, revealing that the deepest branch within Palaeognathae separates the ostrich from other members of the clade, rather than the traditional hypothesis that separates the tinamous from the ratites. To ensure these results reflected evolutionary history, we examined potential biases in types of loci used, heterotachy, alignment biases, and discordance between gene trees and the species tree. All analyses consistently supported nonmonophyly of the ratites and no confounding biases were identified. This confirmation that ratites are not monophyletic using independent evidence will hopefully stimulate further comparative research on paleognath development and genetics that might reveal the basis of the morphological convergence in these large, flightless birds. [alignment bias; convergence; gene tree—species tree discordance; mixture models; paleognath.]

> *"The ratites are a truly natural group. Ostriches, emus, cassowaries, rheas, kiwis, moas and elephant birds really are more closely related to each other than they are to any other birds. And their shared ancestor was flightless too."* –The Elephant Bird's Tale, *Dawkins (2004)*

Advances in sequence data acquisition and bioinformatics have greatly improved the field of phylogenetic estimation, and these advances have led to the publication of a number of large-scale phylogenetic studies (e.g., Dunn et al. 2008; Hackett et al. 2008; Wiens et al. 2008). However, even estimates of phylogeny based upon large-scale data sets may not accurately reflect evolutionary history. For example, the position of the ctenophores in the animal tree of life has differed among several large-scale studies (e.g., Dunn et al. 2008; Hejnol et al. 2009; Philippe et al. 2009; Schierwater et al. 2009). These differences may be due to systematic errors such as long-branch attraction (Felsenstein 1978), convergence in base composition (Phillips et al. 2004; Jeffroy et al. 2006), problems associated with mixture models (Kolaczkowski and Thornton 2004; Matsen and Steel 2007), and gene tree—species tree discordance (Degnan and Rosenberg 2006). Models of sequence evolution that better fit the actual process of evolution have the potential to eliminate the impact of these errors (Steel 2005; Waddell et al. 2009), though currently available models and methods of phylogenetic inference remain imperfect (Thornton and Kolaczkowski 2005). Other factors, including incorrect multiple sequence alignments (Lake 1991) and a variety of issues associated with data quality control (Philippe et al. 2011), can also lead to incorrect but strongly supported estimates of phylogeny. Therefore, novel and controversial phylogenetic results, even those that appear strongly supported by large amounts of data, should continue to be rigorously evaluated.

Many studies addressing difficult phylogenetic problems have focused on using better fitting models of sequence evolution and noise reduction (e.g., Braun and Kimball 2002; Pratt et al. 2009). However, another approach that has substantial potential is the use of independent evidence (i.e., data that were not included in the original study). Independent evidence may be particularly powerful when previous analyses either conflict with strong prior belief or yield conflicting results. However, even when independent evidence is analyzed, it remains critical to examine the data matrix carefully and use analyses that address potential sources of error in tree estimation (Philippe et al. 2011). Examining the results of previous phylogenetic analyses in light of independent evidence can either corroborate the original hypothesis, suggest a modified hypothesis (e.g., Wang et al. 2012), suggest an alternative hypothesis (e.g., Morgan-Richards et al. 2008), or even establish that the original hypothesis was driven by unique characteristics of the original data set.

One controversial relationship suggested by a large-scale multilocus study involves the monophyly, or lack thereof, of ratites (Harshman et al. 2008). Ratites are large, flightless birds of Southern landmasses and they

include some of the most recognizable extant avian taxa like emus, rheas, and ostriches. The name ratite refers to the flat, "raft-like" sternum characteristic of these birds (Merrem 1813). The ratite sternum lacks a keel for attachment of flight muscles, in contrast to a typical carinate sternum that is suited for powered flight. This feature was used to separate extant birds into two groups, Ratitae and Carinatae (Merrem 1813). Huxley (1867) suggested that the flightless ratites are central to understanding the early evolution of birds based upon the existence of these two sternum types. However, a definitive resolution of this question has proven difficult.

Extant ratites are found throughout the Southern hemisphere (excluding Antarctica) and the distribution of these birds on disparate landmasses presented a fundamental biogeographic problem for Huxley's (1867) hypothesis that ratites are a relatively ancient group. In the 19th century, it was unclear how members of a monophyletic group of large flightless birds (Fig. 1a) could be distributed across far-flung southern landmasses. Indeed, the contrasting view, that the ratite sternum reflected convergence due to flightlessness (Fig. 1b), arose at the same time (Owen 1866; Fürbringer 1888; Parker 1895; Wetmore 1930), and became the dominant view for many years (e.g., Mayr and Amadon 1951). The hypothesis that the ratite sternum had multiple origins, rather than being a synapomorphy uniting a monophyletic Ratitae, gained further support when the existence of a plausible developmental explanation (neoteny) for the similarities exhibited by the ratites was noted (McDowell 1948; DeBeer 1956).

The hypothesis that ratites are monophyletic was reinvigorated in the late 20th century by the recognition that continental drift provides a plausible explanation for the distribution of extant ratites. Specifically, the breakup of the Gondwana supercontinent has the potential to explain the distribution of extant ratites (Cracraft 1973). Although none of the proposed phyletic branching patterns for ratites (e.g., Sibley and Ahlquist 1990; Cooper et al. 2001; Haddrath and Baker 2001; Livezey and Zusi 2007; Bourdon et al. 2009) correspond perfectly to the order of separation of landmasses during the breakup of Gondwana (Harshman et al. 2008), the notion that continental drift explains ratite distribution has became a textbook example of vicariance biogeography used for both pedagogical purposes (e.g., Futuyma 2005; Gill 2007) and popular presentations (e.g., Dawkins 2004). Indeed, the hypothesis of ratite monophyly combined with Gondwana-based vicariance biogeography has remained the dominant paradigm of evolutionary history for ratites in recent decades.

The issue of ratite monophyly has often been combined with their placement into a larger clade (Palaeognathae). Paleognaths are united by a specific palate structure (Huxley 1867; Pycraft 1900), and are composed of ratites and tinamous, a volant South and Central American group. Although the early publications describing the paleognathous palate did not provide unambiguous support for paleognath monophyly, later analyses of both morphological (e.g., Bock 1963; Parkes and Clark 1966; Cracraft 1974; Lee et al. 1997; Livezey and Zusi 2007) and molecular data (e.g., Sibley and Frelin 1972; Prager et al. 1976; Stapel et al. 1984; Sibley and Ahlquist 1990; Lee et al. 1997; Braun and Kimball 2002) have supported ratite and/or paleognath monophyly (Fig. 1a). The alternative hypothesis that ratites arose by convergence has been viewed as implying

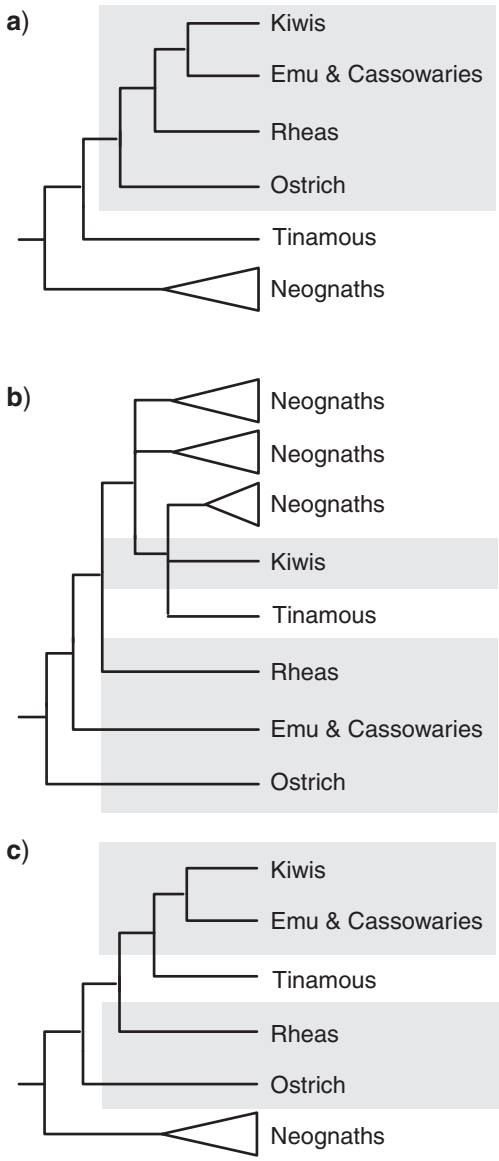

FIGURE 1. *A priori* hypotheses describing ratites and paleognath relationships The flightless ratites shaded in gray. a) Ratite and paleognath monophyly. The topology within ratites corresponds to that found by Sibley and Ahlquist (1990), but other topologies within ratites have been proposed (e.g., Lee et al. 1997; Livezey and Zusi 2007). b) Ratite and paleognath polyphyly. This hypothesis is consistent with many different topologies; the example shown here is from Fürbringer (1888) as summarized by Mayr (2011). c) Monophyly of paleognaths and nonostrich ratites, the topology suggested by recent analyses of nuclear (Chojnowski et al. 2008; Hackett et al. 2008; Harshman et al. 2008) and mitochondrial (Phillips et al. 2010) sequence data.

that neither Ratitae nor Palaeognathae are monophyletic (Fig. 1b), so the accumulation of support for monophyly of both groups has been viewed as falsifying the hypothesis of convergence in the ratite sternum. Equally important, the hypothesis that neoteny has the potential to result in sufficient convergence to be misleading in phylogenetic analyses has never been corroborated (e.g., Gussekloo and Bout 2002). Thus, the hypothesis that similarities among ratites reflect common ancestry (Fig. 1a) appears better corroborated than the hypothesis that the similarities among ratites reflect convergence (Fig. 1b).

Recent studies using nuclear sequence data provide a third alternative (Fig. 1c), supporting paleognath monophyly but rejecting ratite monophyly (Chojnowski et al. 2008; Hackett et al. 2008; Harshman et al. 2008; Yuri et al. 2008). Unlike previous studies, these studies support a clade of nonostrich paleognaths that places the volant tinamous within the flightless ratites. This phylogenetic position suggests that multiple losses of flight led to convergence among the ratites but confirms the ancient divergence of the paleognaths from all other birds. However, these studies were not independent of each other because all used a subset of the data that was examined in detail by Harshman et al. (2008). In contrast, analyses of two nuclear regions not included in Harshman et al. (2008), an intron in the *CHD1* gene (García-Moreno and Mindell 2000) and the *MOS* (c-*mos*) coding region (García-Moreno et al. 2003), both support ratite monophyly (although taxon sampling was limited for *MOS*). Complete mitochondrial genomes do not resolve this issue. Although a recent analysis of complete mitochondria conducted by Phillips et al. (2010) corroborates the Harshman et al. (2008) phylogeny, a number of analyses of mtDNA (including other analyses that used complete mitochondrial genomes) have suggested ratite monophyly (e.g., Lee et al. 1997; Haddrath and Baker 2001; Braun and Kimball 2002; Gibb et al. 2007). It is clear that a phylogenetic signal supporting paleognath monophyly is evident in most studies, but the issue of ratite monophyly remains uncertain.

Given the conflicting hypotheses regarding ratite monophyly and the recognition that even large-scale data sets like Harshman et al. (2008) have the potential to be misleading, we collected a novel 40-locus data set. These loci comprise more than 22 kilobases (kb) of nuclear sequence data located throughout the avian genome, none of which have been used in any previous study of paleognath phylogeny. In addition, we examined whether bias in locus selection, incorrect multiple sequence alignments, and mixture models (accounting for heterotachy and gene tree–species tree discordance) impact our estimation of paleognath phylogeny. These analyses complement Harshman et al. (2008), who examined and rejected the possibility that either long-branch attraction or base composition convergence affected their conclusions. Because other large-scale studies have obtained conflicting results even when partially overlapping data sets were examined (e.g., Dunn et al. 2008; Hejnol et al. 2009; Philippe et al. 2009; Schierwater et al. 2009), we reasoned that these analyses of a completely independent large-scale data set would provide a rigorous test of the phylogenetic relationships among ratites and other paleognaths.

Methods

Data Collection

We selected 10 taxa appropriate for testing ratite monophyly (Supplementary Table 1, available from Dryad data repository; http://datadryad.org, doi:10.5061/dryad.5vd2560f, last accessed August 7, 2012) and collected data from a total of 40 loci (Supplementary Table 2) that were distinct from those used in Harshman et al. (2008) and other studies that include sufficient taxa to test ratite monophyly (i.e., García-Moreno and Mindell 2000; García-Moreno et al. 2003). Mapviewer, available from the NCBI website (http://www.ncbi.nlm.nih.gov/mapview, last accessed August 7, 2012), was used to establish the position of each locus in the chicken genome, showing that the loci were distributed throughout the chicken genome and are unlikely to be linked (Supplementary Table 2). Locus names were obtained from the HUGO Gene Nomenclature Committee (HGNC) database (http://www.genenames.org, last accessed August 7, 2012; Eyre et al. 2006).

Locus Development

Two selection strategies were used to select loci for this project: (i) the exon-primed intron crossing (EPIC) approach (Palumbi and Baker 1994) that targeted identified loci *a priori* and (ii) an anonymous approach (e.g., Karl and Avise 1993; Jennings and Edwards 2005; Thomson et al. 2008) that selected regions randomly (and thus had the potential to access different regions of the genome). The majority of loci for this study were selected using the EPIC approach. This included 12 loci (from Cox et al. 2007; Kimball et al. 2009) that have been published previously but have not been used to examine paleognath phylogeny, and 18 loci developed for use in this study (Supplementary Table 2). Novel primers were designed by comparing sequences from the chicken genome and other available avian data for primer design. BLAT (Kent 2002) was used to rapidly identify exon boundaries and assess intron length, facilitating primer design in the coding regions. Primers were designed to isolate short introns (e.g., 500 bp) that do not require internal primers. Primers and introns were numbered using the protocol of Kimball et al. (2009).

Ten anonymous loci were used in this study; anonymous loci represent arbitrary fragments of the genome and can provide data from large introns and intergenic regions that are not available using the EPIC approach. It is unclear whether the phylogenetic signal in regions like long introns or intergenic regions

differs from the nuclear regions (exons, short introns, and 3′-untranslated regions) used by Harshman et al. (2008), but use of these alternative regions allowed us to explore this possibility. In order to isolate the random genomic regions necessary for anonymous locus development, a small insert nuclear DNA library was constructed from the little tinamou. To construct this, genomic DNA was sheared via sonication to produce fragments ∼2 kb in length. Fragments were blunt end repaired via the DNA Terminator® End Repair Kit (Lucigen® Corporation) and cloned using the pEZSeq™Blue/White Cloning Kit (Lucigen® Corporation) for high efficiency cloning. Plasmids were prepared for sequencing by TempliPhi purification (Amersham Biosciences). Clones were selected randomly from the little tinamou library and sequenced as described below. Sequences from 304 clones were compared with the chicken genome using BLASTN searches to identify regions of homology. Primers were designed from 39 nonrepetitive, homologous regions greater than 300 bases in length. Of these, primers from 10 anonymous loci amplified robustly enough to be used for this study: Four intergenic regions, three regions containing relatively large proportions of both intronic and exonic sequence, two from large introns, and one coding region (Supplementary Table 2).

It is critical for large-scale phylogenetic studies to ensure that all loci used are likely to represent orthologous sequences and do not contain obvious errors (e.g., Philippe et al. 2011). To accomplish this, we carefully examined gene trees for individual loci, sequences that appeared problematic were reamplified and sequenced. One locus that appeared likely to include paralogs (PCR products obtained by repeated amplifications of *Rhea* and *Pterocnemia*, which are closely related sister taxa, corresponded to divergent sequences that were not monophyletic) was excluded from this study.

Amplification and Sequencing

We used standard methods for PCR amplification (Harshman et al. 2008; Kimball et al. 2009). Most PCR products were cleaned by PEG:NaCl (20%:2.5 M) precipitation, although PCR reactions that produced multiple bands were purified using the Perfectprep® Gel Cleanup kit (Eppendorf) after excision of the desired amplicon from an agarose gel. To obtain unambiguous sequences of PCR products that were derived from individuals that were heterozygous for alleles of different lengths, amplicons were cloned into pGEM®-T Easy (Promega). PCR products and plasmids were sequenced using the amplification primers. Sequences were obtained using an ABI Prism™ 3100-Avant genetic analyzer (PE Applied Biosystems) with ABI BigDye® Terminator v.3.1 chemistry. Sequencher™ 4.1 (Gene Codes Corp.) was used to edit sequences and assemble double-stranded contigs.

Sequence Alignment

We applied a manual alignment strategy as described previously (Chojnowski et al. 2008; Hackett et al. 2008; Harshman et al. 2008; Kimball and Braun 2008). Regions where alignment was problematic and homology of sites was ambiguous were excluded from analyses, as done by Hackett et al. (2008). In addition, the short flanking exons of EPIC loci were also excluded from analyses. We used the manual alignments for all analyses except those aimed at testing the sensitivity of our conclusions to alignment bias.

To specifically evaluate alignment bias, we also used an automatic alignment strategy that employed the progressive alignment program PRANK (the Probabilistic Alignment Kit; Löytynoja and Goldman 2005). For the PRANK alignments, we used the entire locus (no sites were excluded) and 15 different guide trees that represent all plausible topologies for the taxa examined here. The set of 15 plausible topologies constrain both tinamous and rheas to be monophyletic and fixes the outgroup topology (the outgroup taxa were chosen because they have a topology that was not controversial; see Cracraft et al. 2004; Hackett et al. 2008). Branch lengths for the guide trees were estimated for each locus by maximum likelihood (ML) using PAUP* 4.0b10 (Swofford 2003) and the manual alignments. Then each of the 15 guide trees (with appropriate branch lengths for each locus) was used to generate alignments for all loci. These alignments were combined to produce a total of 15 concatenated data sets with 40 loci each. These PRANK alignments were used only to evaluate the sensitivity of results to sequence alignment bias; other analyses used the manual alignments because they were not produced using any specific guide tree.

Phylogenetic Analyses

We conducted a set of analyses on the independent evidence data set to not only investigate paleognath relationships but to also ascertain confidence in the resulting phylogenies and examine the source of any misleading conclusions using the manual alignment.

Data sets with large numbers of sites have greater power to resolve difficult phylogeneic problems (e.g., Chojnowski et al. 2008), suggesting that analyses of concatenated data will be useful as long as the species tree does not fall into a problematic part of parameter space (within or near the anomaly zone; Kubatko and Degnan 2007). Therefore, we used PAUP* 4.0b10 to conduct concatenated maximum parsimony (MP) and ML analyses of the complete, 22-kb data set, as well as separate anonymous and EPIC partitions, using heuristic searches with 10 random addition replicates and TBR (tree-bisection–reconnection) branch swapping. MP and ML analyses of individual loci were also conducted using branch and bound searches. The appropriate model for ML analyses of individual loci and the concatenated alignments was determined using

the Akaike information criterion (AIC) to choose the best fitting model from the set of models examined by MODELTEST 3.7 (Posada and Crandall 1998). To improve model fit, we also conducted partitioned ML analyses of the multilocus data sets using RAxML 7.2.8a (Stamatakis 2006) with GTRGAMMA and considering each locus as a partition. We also compared the fit of partitioned and unpartitioned analyses in RAxML using the AIC. Finally, we conducted 1000 bootstrap replicates for both MP and ML analyses (in PAUP* using the conditions described above) and partitioned ML analyses (in RAxML using GTRCAT).

To determine whether our 40-locus data set could reject some alternative topologies, we used the approximately unbiased (AU) test (Shimodaira 2002) as implemented in PAUP* 4.0a122 (all other analyses used PAUP* 4.0b10) using the best-fitting model for the complete data matrix (GTR+Γ+inv sites). The AU test provides information about the set of topologies that are plausible at a certain confidence level. The set of trees we examined using the AU test comprised 15 trees (these were identical to the plausible set of trees corresponded used as guide trees for PRANK alignments, see above).

The concatenated alignments generated using PRANK and each of the 15 guide trees (hereafter, these are called "the PRANK alignments") were analyzed in PAUP* using ML. The GTR+Γ+inv sites model was used to analyze each of the 15 concatenated PRANK alignments. Support for specific clades in analyses of the PRANK alignments was assessed using 1000 bootstrap replicates as described above.

To complement the MP and ML analyses, Bayesian Markov chain Monte Carlo (MCMC) analyses were conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) and BayesPhylogenies 1.1 (Pagel and Meade 2004). Concatenated analyses of all data matrices, including the 40-locus data set, the total evidence data set, and separate anonymous and EPIC data sets were conducted in MrBayes and were partitioned by locus. The appropriate model for each locus was determined using the AIC (limiting the set of models under consideration to those implemented in MrBayes). MrBayes analyses used two simultaneous searches with four chains each (three heated chains and one cold chain) that were run for 50 million generations, sampling every 1000 generations and discarding the first 5 million generations as "burn-in." We ensured that runs had converged by noting that the harmonic means of the different runs were similar, that the posterior scale reduction factors were essentially 1, and that the average deviations of the split frequencies were substantially smaller than 0.01.

When there is heterotachy, or changes in evolutionary rates at specific sites over time, some methods of phylogenetic reconstruction can fail due to mixed branch repulsion (Kolaczkowski and Thornton 2004; Gadagkar and Kumar 2005; Gaucher and Miyamoto 2005). In the extreme, Matsen and Steel (2007) demonstrated that a mixture of trees with distinct sets of branch lengths could result in the same expected site pattern frequencies as a tree with a different topology. To examine the potential influence of heterotachy upon our conclusions, we conducted Bayesian analyses of the 40-locus data set using models that accommodate those changes. Two different approaches were used: (i) the covarion model implemented in MrBayes (run as described above for the partitioned analysis in MrBayes) and (ii) the branch lengths mixture approach implemented in BayesPhylogenies. The BayesPhylogenies analyses used a single chain that was run for 50 million generations, sampling every 10 000 generations and discarding the first 5 million generations as burn-in. To assess convergence, we conducted three independent BayesPhylogenies analyses; all of the analyses provided similar results.

Multilocus data typically reflect a mixture of discordant gene trees as well. The most frequent gene tree may not correspond to the true species tree in some instances (Degnan and Rosenberg 2006), a discrepancy that can mislead concatenated analyses (Kubatko and Degnan 2007). However, the observation that individual estimates of gene trees exhibit incongruence does not establish that the gene trees have different evolutionary histories. To determine whether discordance among gene trees had an impact upon our concatenated analyses, we used BUCKy 1.4.0 (Ané et al. 2007) to estimate the proportion of the genome that supports a given clade using a Bayesian framework. The trees for each of the 40 individual loci that were used for this concordance analysis were generated using MrBayes with model selection and run criteria as described above. We note that conducting analyses of independent evidence has the benefit of allowing the use of published studies to obtain good estimates of priors in addition to providing a source of information that can corroborate phylogenetic conclusions. Using the information from Harshman et al. (2008) and Phillips et al. (2010), we set the BUCKy gene tree discordance prior (α) to a value of 3.0.

We used BUCKy 1.4.0 (Larget et al. 2010) to provide an estimate of the population (species) tree obtained using a quartet method. As long as concordance factors are estimated accurately this quartet method is a consistent estimator of the true species tree (Degnan et al. 2009). A second approach that we used to evaluate the impact of incongruence among gene trees was to examine the ML and MP estimates of trees for individual partitions. We divided gene trees into two classes: Those with a nonostrich paleognath clade and those without. We calculated the maximum expected number of trees in each category given the null hypothesis that the nonostrich paleognath clade was not present in the species tree (for details see Supplementary Fig. 1). Then we used a χ^2 test to determine whether the observed number of individual gene trees with this topology exceeded the number expected given that null hypothesis.

Deviations from base compositional stationarity have been shown to have an impact on phylogenetic estimation (e.g., Jeffroy et al. 2006). We used two different

approaches to look for changes in base composition over evolutionary time. First, PAUP* was used to calculate base composition for variable sites in each locus and those values were used to calculate the relative composition variability (RCV; Phillips and Penny 2003) as a summary statistic. Second, the χ^2 test of base composition implemented in PAUP* was used to identify loci that showed significant deviation from stationarity.

Finally, we conducted total molecular evidence analyses by concatenating the 40-locus data set obtained for this study with the published 20-locus data set of Harshman et al. (2008) and the mitochondrial data of Phillips et al. (2010). For the total evidence analyses, we used the outgroups included in this study; the outgroup sequences were obtained from a number of different publications (Desjardins and Morais 1990; Pereira et al. 2002; Sorenson et al. 2003; Pereira and Baker 2004; Mossman et al. 2006; Hackett et al. 2008; Warren et al. 2010; Braun et al. 2011). We conducted analyses using: (i) the ingroup species included in this study and (ii) the ingroup taxa used by Phillips et al. (2010) which included three extinct moas (*Emeus*, *Anomalopteryx*, and *Dinornis*) and two additional species of kiwi (*Apteryx haastii* and *A. owenii*); the moas and additional kiwis were only represented by mitochondrial data. Following Phillips et al. (2010), we RY-coded mitochondrial third codon positions. For both taxon sets, we conducted unpartitioned and partitioned ML and bootstrap ML analyses, MP and MP bootstrap analyses, and Bayesian analyses as described above. The data were partitioned by locus for nuclear data and into four partitions (one for each codon position and one for the noncoding sequences) for mitochondrial data.

All sequences collected from this study have been deposited in GenBank (Accession Numbers JX120800-JX121088). Alignments and trees have been deposited in TreeBASE (Submission ID 12891) and they are also available from the "Early Bird" website (http://www.biology.ufl.edu/earlybird, last accessed August 7, 2012).

Results

Does Independent Evidence Support Monophyly of Nonostrich Paleognaths?

All analyses of the 40-locus concatenated data set, including analyses partitioned by locus, collected for this study indicate that the deepest divergence within extant paleognaths separates the ostrich from all nonostrich paleognaths, including the volant tinamous (Fig. 2). When examining all 15 plausible paleognath topologies, there was only a single tree found in the 95% credible set of trees and it supported nonostrich paleognath monophyly (Table 1). The AU test indicated that only two topologies, both of which support nonostrich paleognath monophyly, could explain the data at the $P < 0.05$ level (Table 1). Moreover, we note that the topologies that failed to support monophyly of nonostrich paleognaths, including the three topologies

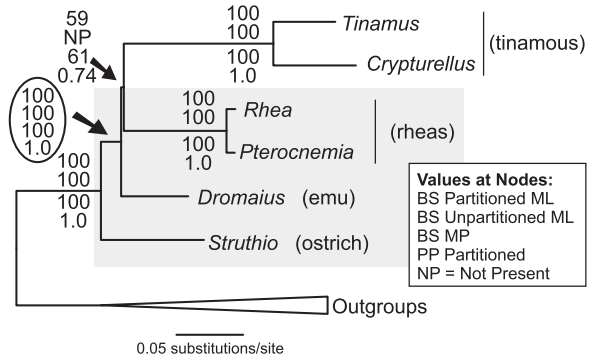

FIGURE 2. Maximum likelihood phylogram from a partitioned analysis with support from multiple analyses (BS refers to % bootstrap support, PP refers to posterior probability values). The ratites are shaded in gray. Partitioning by locus had a much better fit to the data than unpartitioned analyses ($AIC_{partitioned}$ = 198 500.03, $AIC_{unpartitioned}$ = 200 398.14, Δ_{AIC} = 1898.11).

that are consistent with ratite monophyly, could be strongly rejected using the AU test and excluded from the 99% credible set established by the MrBayes analysis. These results corroborate Harshman et al. (2008), the previous studies that were based upon subsets of the nuclear gene regions used in that study (Chojnowski et al. 2008; Hackett et al. 2008; Yuri et al. 2008), and the reanalyses of mitochondrial data presented by Phillips et al. (2010).

Does accounting for heterogeneity suggest ratite monophyly?—The phylogenetic results of Harshman et al. (2008) were based upon the concatenation of multiple loci, as were those of Chojnowski et al. (2008) and Hackett et al. (2008). Although Harshman et al. (2008) conducted partitioned analyses of the data that allow model parameters to vary among loci, it is unclear whether other types of among-partition heterogeneity had an impact on our estimate of phylogeny. The underlying processes that generate specific sets of site patterns in concatenated multiple sequence alignments are expected to be heterogeneous (e.g., Edwards 2009). Types of heterogeneity that are often considered include processes that result in: (i) a mixture of distinct sets of branch length on a single tree and (ii) a mixture of gene trees with distinct topologies.

To examine the first source of heterogeneity, we used the covarion model implemented in MrBayes and the branch length mixture model implemented in BayesPhylogenies because the best phylogenetic method for analyses of heterotachous data remains unclear (Zhou et al. 2007; Kolaczkowski and Thornton 2008). Both approaches strongly supported monophyly of the nonostrich paleognaths (posterior probabilities of 1.0 in both cases). Moreover, the branch length mixture model indicated that most branches did not exhibit evidence of heterogeneity (i.e., a single branch length was typically sampled by the MCMC chain), further suggesting that heterotachy is unlikely to have a major impact on our estimate of phylogeny.

TABLE 1. Support for each of the 15 plausible topologies for Palaeognathae

Topology number	Topology	AU test *P*-value	Bayesian posterior probability
1	(Outgroup,((Rheas,(Tinamous,Emu)),Ostrich))	MLE	0.967
2	(Outgroup,(((Rheas,Tinamous),Emu),Ostrich))	0.084	0.027
3	(Outgroup,(((Rheas,Emu),Tinamous),Ostrich))	0.029	0.005
4	(Outgroup,(((Rheas,Tinamous),Ostrich),Emu))	0.000	0.000
5	(Outgroup,(((Rheas,Ostrich),Tinamous),Emu))	0.000	0.000
6	(Outgroup,((Rheas,(Tinamous,Ostrich)),Emu))	0.000	0.000
7	(Outgroup,(Rheas,((Tinamous,Emu),Ostrich)))	0.000	0.000
8	(Outgroup,(Rheas,(Tinamous,(Emu,Ostrich))))	0.000	0.000
9	(Outgroup,(Rheas,((Tinamous,Ostrich),Emu)))	0.000	0.000
10	(Outgroup,((Rheas,Tinamous),(Emu,Ostrich)))	0.000	0.000
11	(Outgroup,((Rheas,Emu),(Tinamous,Ostrich)))	0.000	0.000
12	(Outgroup,((Rheas,Ostrich),(Tinamous,Emu)))	0.000	0.000
13	(Outgroup,(((Rheas,Emu),Ostrich),Tinamous))	0.000	0.000
14	(Outgroup,((Rheas,(Emu,Ostrich)),Tinamous))	0.000	0.000
15	(Outgroup,(((Rheas,Ostrich),Emu),Tinamous))	0.000	0.000

Bayesian posterior probability refers to the probability of the entire topology, not a single node. Topologies 1–3, where ostriches are sister to other paleognaths, are the best-supported topologies. MLE = maximum likelihood estimate.

To examine the second source of heterogeneity (i.e., gene tree–species tree discordance), we identified the primary concordance tree using BUCKy. The genome-wide concordance factor suggests approximately 93% of the paleognath genome (95% credible interval of 84–99%) supports monophyly of the nonostrich paleognaths (Fig. 3). The estimates of concordance factors were virtually identical to those obtained for the outgroups (Fig. 3), where phylogenetic relationships are not controversial.

An examination of the individual loci provides further support. Excluding two loci that lacked one extant paleognath lineages, both ML and Bayesian analyses of individual loci indicated that the majority of loci [24 of 38 (63%) loci in both cases] supported nonostrich paleognath monophyly (Table 2). The number of individual loci expected to support nonostrich paleognath monophyly if the species tree lacks a nonostrich paleognath clade is <16% (Supplementary Fig. 1); our data strongly reject (χ^2 = 62.88; df = 1; $P < 10^{-14}$) the hypothesis that the species tree lacks a nonostrich paleognath clade. The results of MP analyses were similar, although they showed slightly less support for nonostrich paleognath monophyly (15 of 38 loci supported this topology; Table 2). This could reflect the greater sensitivity of MP to phenomena like long-branch attraction, but even if the MP topologies for individual gene trees are assumed to be correct the null hypothesis could be strongly rejected (χ^2 = 15.58; df = 1; $P < 10^{-4}$). Given these analyses, it seems unlikely that gene tree—species tree discordance can explain our conclusions of nonostrich paleognath monophyly; instead, it seems more likely that the observed incongruence among individual gene trees reflects mutational error (Huang et al. 2010). It is clear that mutational error can have a major impact when short regions of individual loci are sampled (Chojnowski et al. 2008), as we did for this study.

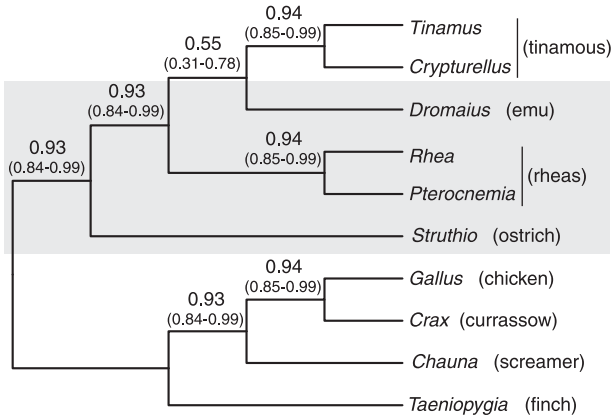

FIGURE 3. Species tree estimated using BUCKy (which was topologically identical to the primary concordance tree). The flightless ratites are shaded in gray. The top values are concordance factors (the estimated amount of the genome supporting a particular bipartition); the lower values provide the 95% credible intervals for the concordance factor the estimates.

Cases in which the species tree falls within the anomaly zone are expected to favor symmetric gene trees even when the species tree is asymmetric (Degnan and Rosenberg 2006). Of the 35 trees that could be categorized as symmetric or asymmetric (five could not be categorized this way due to polytomies or missing taxa), only a single ML gene tree (EIF5) had a symmetric topology (Supplementary Table 3). In fact, the second most common topology following nonostrich paleognath monophyly was an asymmetric tree with rheas sister to all other paleognaths (observed for five loci) (Supplementary Table 3).

Do certain types of loci suggest ratite monophyly?—There do not appear to be obvious biases in the loci that could explain the strong support found

TABLE 2. Characteristics and performance of different partitions

Locus	RCV	Support for nonostrich paleognath monophyly		
		ML	MP	PP
EPIC		100	100	1.0
ACTB	0.0695	100	99	1.0
ARNTL	0.0844	86	88	0.93
CALB1	0.0795	69	–	0.77
CHMP5	0.0618	–	–	–
CIZ1	0.0664	53	38	0.71
CLOCK	0.0696[a]	72	74	1.0
CRAT	0.1005[a]	99	88	1.0
CSDE1	0.0579	72	–	0.85
CSNK1E	0.0726	66	−(81)	0.68
DDX5	0.0609	–	–	–
EIF5	0.0926	–	–	–
ENO1	0.0935	74	76	0.97
ETS2	0.0832	86	–	0.98
GAPDH	0.0664	−(50)	−(75)	−(0.60)
GNB2L1	0.1073	49	68	0.57
GRIA2	0.0768	43	–	0.46
HNRPA2B1	0.0584	−(45)	−(53)	−(0.42)
KCNQ5	0.0507	68	–	0.89
NAT15	0.0721	–	–	–
PARK7	0.0453	90	89	1.0
PAXIP1	0.0634	–	–	–
PER2	0.0673	67	−(46)	0.90
PHB	0.0625	–	–	–
PSMA2	0.0523	51	−(51)	0.56
SEPT2	0.0540	73	89	0.82
SFRS3	0.1378[a]	96	93	0.99
TCP1	0.1180	–	–	–
TXNDC12	0.0886	80	86	0.82
VDAC2	0.0614	–	−(69)	–
VIM	0.0675	52	57	0.71
Anonymous		100	90	1.0
BMP5	0.1025	−(25)[b]	–	–
Intergenic 1	0.0876	94	89	1.0
Intergenic 2	0.0875	83	–	0.99
Intergenic 3	0.0724	69	69	0.89
Intergenic 4	0.2514[a]	−(63)	−(92)	−(0.92)
NUSAP1	0.0871	–	–	−(0.31)
PALLD	0.1121[a]	84	85	0.99
PUM2	0.0738	73	50	0.81
SLC25A21	0.0966[a]	–	–	–
TTN	0.1030	46	64	0.46
Published		100	79	1.0
ALDOB	0.0596[a]	52	66	0.92
BDNF	0.3071[a]	–[c]	–	–
CLTC	0.0457	–	–	–
CLTCL1	0.0629	−(69)	72	0.95
CRYAA	0.1410[a]	68	80	0.96
EEF2	0.0873[a]	−(53)	90	1.0
EGR1	0.1108[a]	–	–	0.65
FGB	0.0281	71	63	0.86
GH1	0.0801[a]	−(64)	44	0.53
HMGN2	0.1146[a]	−(66)	−(43)	−(0.46)
IRF2	0.0769	47	53	0.59
MB	0.0721	91	99	1.0
MUSK	0.0866[a]	99	98	1.0
MYC	0.1170[a]	−(53)	77	0.97
NGF	0.2320[a]	–[c]	–[c]	–[c]
NTF3	0.1220[a]	−(58)	65	0.92
PCBD1	0.1029[a]	−(65)	90	1.0
RHO	0.0980[a]	–	77	0.95
TGFB2	0.0440	–	–	–

Continued

TABLE 2. Continued

Locus	RCV	Support for nonostrich paleognath monophyly		
		ML	MP	PP
TPM1	0.0933	–	–	–
Mitochondrial	–[d]	–(98)	–(64)	–(1.0)

Numbers are the percent bootstrap support or posterior probabilities. A dash indicates the extended majority rule bootstrap consensus topology did not support nonostrich paleognath monophyly; numbers in parentheses following dashes indicate bootstrap support for the conflicting hypothesis of ratite monophyly. RCV = relative composition variability.

[a]Indicates locus with significant ($P < 0.05$) deviation from base compositional stationarity based upon the χ^2 test implemented in PAUP*.

[b]The ML topology for BMP5 is unresolved, but the extended majority rule consensus tree has 23% support for ratite monophyly.

[c]These analyses did not support paleognath monophyly.

[d]Mitochondrial base composition was examined for each partition (codon positions and noncoding regions). RCV values: pos1 = 0.0859; pos 2 = 0.0439; pos 3 = 0.0260; NC = 0.0642. Pos1 and NC exhibited a significant deviation from stationarity. Pos3 was subjected to RY-coding, similar to Phillips et al. (2010).

for nonostrich paleognath monophyly (Table 2 and Supplementary Table 3). Analyses of concatenated alignments that corresponded only to the EPIC or the anonymous loci both strongly support nonostrich paleognath monophyly in both ML and MP analyses (Table 2 and Supplementary Table 3), consistent with the interpretation of the BUCKy results as a genome-wide signal (Fig. 3). In addition, equal proportions of EPIC and anonymous loci support nonostrich paleognath monophyly (Table 2 and Supplementary Table 3). There were no major differences in either the base composition or RCV between those loci that supported nonostrich paleognaths and those that did not, or between EPIC and anonymous loci in general (Table 2). Given the similar phylogenetic signal and parameters among different loci, and the greater difficulty of obtaining data from anonymous loci (see above in Methods), our results suggest either EPIC or anonymous loci are appropriate, though the difficulties of working with anonymous loci may make it more advantageous to focus on EPIC loci in future studies. Taken as a whole, these analyses of individual loci strongly corroborate the hypothesis that there is a strong genome-wide signal that supports nonostrich paleognath monophyly.

Does the use of different sequence alignments suggest ratite monophyly?—Biases capable of misleading phylogenetic analyses can be introduced into alignments (e.g., Lake 1991; Nelesen et al. 2008). This has the potential to be especially problematic when the majority of data examined are noncoding, as they are for this study. To examine this, we used the 15 plausible topologies for our taxon sample (three of which included the conflicting hypothesis of ratite monophyly) as guide trees for PRANK to generate 15 different alignments. Consistent with the existence of a strong signal supporting monophyly of nonostrich paleognaths, the ostrich emerged as the sister of all other paleognaths for all 15 alignments with 100% ML bootstrap support (Table 3). Therefore, the phylogenetic signal supporting ratite polyphyly does not appear to be driven by alignment biases because it emerges even in alignments based on guide trees that assume ratite monophyly.

TABLE 3. Results of analyses using PRANK alignments

Guide tree topology	Estimated ML tree	Bootstrap (%) for nonostrich paleognath monophyly	Bootstrap (%) for tinamou sister relationship
1	1	100	99
2	2	100	99
3	1	100	84
4	2	100	100
5	1	100	93
6	3	100	42
7	1	100	99
8	1	100	100
9	1	100	63
10	2	100	100
11	3	100	99
12	1	100	100
13	1	100	79
14	1	100	87
15	1	100	95

Numbers for guide tree topology and estimated ML tree match those in Table 1. Bootstrap values are from unpartitioned ML analyses.

What Does Total Evidence Suggest About Paleognath Phylogeny?

Combining the data collected for this study with the data from Harshman et al. (2008) and Phillips et al. (2010) results in strong support for separating the ostrich from all other paleognaths, including the tinamous (Fig. 4). However, the addition of both taxa and sequence still cannot resolve the sister group of the tinamous (Fig. 4). In agreement with Phillips et al. (2012), the sister group of the tinamous was the extinct moas (the moas are only represented by mitochondrial data), though uniting the tinamou–moa clade with the rheas receives limited support (Fig. 4a). Analyses using the smaller taxon set resulted in a tinamou–emu clade, but also with limited support (Fig. 4b). Overall, different analytical approaches (e.g., the use of partitioned vs. unpartitioned models) supported different topologies. Most analyses place the tinamous as either sister to the emu (Figs. 2 and 4b) or sister to the rheas (Fig. 4a).

Unlike the position of the ostrich, the sister group of the tinamous is sensitive to alignment (Table 3). All three alignments based on guide trees that had a rhea–tinamou

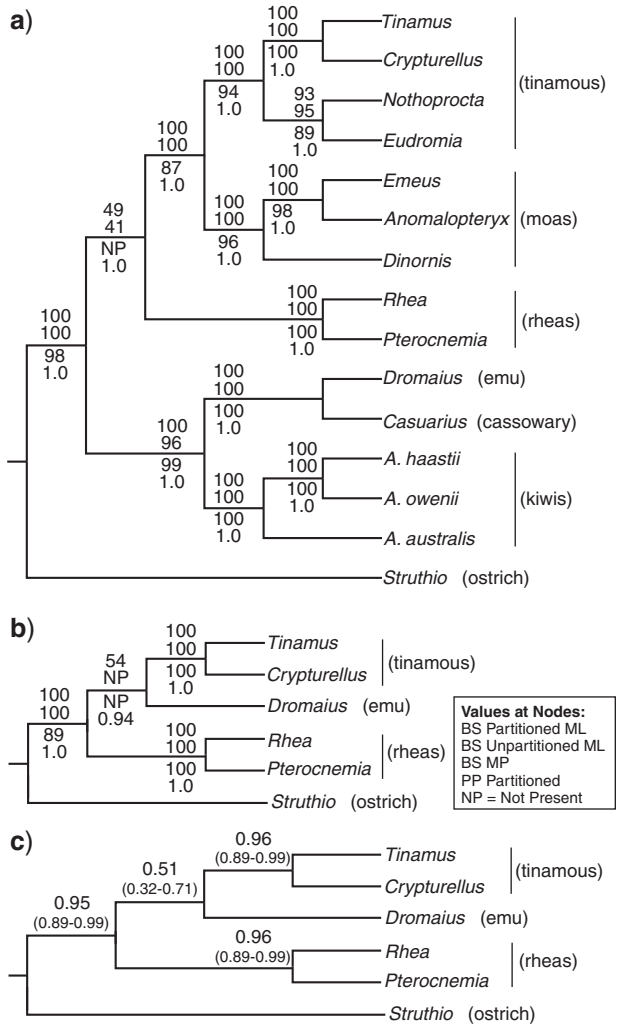

FIGURE 4. Total molecular evidence analysis, concatenating the data from this study with that of Harshman et al. (2008) and Phillips et al. (2010). Support from multiple analyses is presented (BS refers to % bootstrap support, PP refers to posterior probability values). a) ML phylogram for analyses of all paleognath taxa analyzed by Phillips et al. (2010). The Bayesian consensus tree had a different topology, uniting the kiwis, emu, cassowary, moas, and tinamous to the exclusion of the rheas (posterior probability = 0.54). b) ML phylogram for analyses of taxa used in this study. The unpartitioned ML and MP analyses united tinamous and rheas with 51% BS (unpartitioned ML) and 87% BS (MP). c) Species tree estimated using BUCKy for all 61 loci (which was topologically identical to the primary concordance tree). The top values are concordance factors (the estimated amount of the genome supporting a particular bipartition); the lower values provide the 95% credible intervals for these concordance factor estimates. The species tree estimated by BUCKy was topologically identical.

clade resulted in a rhea–tinamou clade, indicating that guide tree bias might have an impact upon estimates of phylogeny. However, for the 12 alignments that were not based on a guide tree that had a rhea–tinamou clade, the majority had an emu–tinamou clade, even when the guide trees lacked an emu–tinamou clade. Thus, the preponderance of evidence based upon the alignment analyses supports an emu–tinamou clade, a finding consistent (given our taxon sampling) with several previous studies (Chojnowski et al. 2008; Hackett et al. 2008; Phillips et al. 2010).

The BUCKy analyses emphasize the difficulty of resolving the sister group of the tinamous. Analyses of the total evidence 61-locus data matrix indicated that just over half of the genome supports the emu–tinamou clade (Fig. 4c). However, the 95% credible intervals of the concordance factor estimates at this node were very wide and included the value expected given a hard polytomy. The concordance factor for uniting the tinamous with the rheas was not much lower (0.44) and the 95% credible intervals (0.25–0.64) overlapped that of the tinamou–emu clade (Fig. 4c). Surprisingly, however, the third possible tree with a nonostrich paleognath clade (tinamous sister to an emu–rhea clade) was not present in the 95% credible set for any locus in the joint estimates of the gene tree topologies.

DISCUSSION

Independent Evidence, Corroboration, and Paleognath Phylogeny

Corroboration has long been viewed as fundamental to the historical sciences, and the observation that estimates of phylogeny obtained using distinct genes show greater similarity than expected by chance has been viewed as a rigorous test of the theory of common descent (e.g., Penny et al. 1982). However, the approach of comparing estimates of gene trees based upon individual loci may not be effective unless each of the loci has sufficient power to resolve the relevant portions of the gene tree correctly (Chojnowski et al. 2008). For many phylogenetic problems, large-scale multilocus data sets (e.g., Hackett et al. 2008) are now used because individual loci lack sufficient power to provide resolved, well-supported estimates of phylogeny. However, even the results of multilocus data sets may be driven by one or two data partitions (e.g., Fig. 1 in Hackett et al. 2008). Therefore, it remains possible that the phylogenetic signals present in those individual loci do not reflect the species tree, due to factors such as gene tree—species tree discordance and patterns of molecular evolution that result in biased estimates of phylogeny for that locus. Therefore, collection of independent evidence provides a potentially important additional tool for systematists that can be used to evaluate controversial phylogenetic hypotheses rigorously.

Independent evidence for monophyly of the nonostrich paleognaths is important given the existence of information favoring the alternative hypothesis of ratite monophyly (e.g., see Cracraft et al. 2004 for review). Indeed, the support for ratite monophyly evident in previous analyses of morphological (e.g., Cracraft 1974; Lee et al. 1997; Livezey and Zusi 2007) and molecular (e.g., Sibley and Ahlquist 1990; Lee et al. 1997; García-Moreno and Mindell 2000; van Tuinen et al. 2000; García-Moreno et al. 2003; Gibb et al. 2007) data could be viewed as a strong prior

in favor of that hypothesis, especially when it is considered in combination with the proposal that vicariance due to the breakup of Gondwana can provide an explanation for ratite biogeography (Cracraft 2001). Although the reanalyses of mitochondrial data presented by Phillips et al. (2010) corroborate the Harshman et al. (2008) hypothesis, mitochondrial sequence data in birds exhibit strong deviations from compositional stationarity (Haddrath and Baker 2001; Braun and Kimball 2002) and phylogenetic analyses of mitochondria are extremely sensitive to both model selection and taxon sampling (Braun and Kimball 2002). Moreover, the mitochondrion reflects a single gene partition so it would be impossible to detect any gene tree—species tree discordance. Thus, it is important to further assess the support for various answers to this question before rejecting the earlier studies demonstrating that ratites are monophyletic.

Our independent evidence corroborating the hypothesis that nonostrich paleognaths form a clade gave us confidence that the still controversial results of Harshman et al. (2008) and Phillips et al. (2010) are unlikely to reflect any specific (and potentially nonhistorical) signal in those data sets. Our analyses consistently support the hypothesis that the deepest-branch within the paleognaths separates the ostrich from all other extant members of the clade, indicating that the volant tinamous are nested inside the other ratites as part of a nonostrich paleognath clade. Additionally, we demonstrated that the signal supporting this conclusion is genome-wide and that the estimates of phylogeny that drive our conclusions did not reflect analytical artifacts and biases. Thus, there are now three large, independent data sets (Harshman et al. 2008; Phillips et al. 2010, and this study) that provide strong support for ratite nonmonophyly.

Nonostrich Paleognaths: Hard Polytomy or Soft Polytomy?

Although the ostrich was consistently placed at the base of the paleognath tree, the sister group to the tinamous differed among analyses. Moreover, the internal branches near the base of the nonostrich paleognaths were very short (Fig. 2). Two hypotheses predominated in the results of our analyses of the novel data set, a clade comprising tinamous and the extant Australasian ratites (the emu, kiwis, and cassowaries) and a tinamou–rhea clade. Most previous studies of both nuclear (Chojnowski et al. 2008; Hackett et al. 2008) and mitochondrial data (Phillips et al. 2010) support a tinamou–Australasian clade. This hypothesis also has some morphological support because the losses of two specific manual phalanges represent potential synapomorphies (Maxwell and Larsson 2009). The second predominant hypothesis, a tinamou–rhea clade, is the most plausible hypothesis from a biogeographic standpoint because both taxa are South American. Resolution was not greatly improved by increasing the size of data set using total molecular evidence analyses (Fig. 4), although there was a slight trend for a tinamou–rhea clade when the most extensive taxon sample was examined (Fig. 4a). However, a simple biogeographic scenario cannot be reconciled with the fact that, like Phillips et al. (2010), the total evidence analysis indicated support for a clade comprising tinamous and the extinct Australasian moas. Support for the tinamou–moa clade is very high, indicating that the Australasian ratites are paraphyletic regardless of the resolution for the extant taxa.

The third possible topology for the nonostrich paleognaths, a rhea–Australasian ratite clade that excludes tinamous, was not supported by any analysis of the independent evidence or the total molecular evidence (Figs. 2 and 4). This third option has been suggested by a morphological analysis (Johnston 2011), but that topology is in strong conflict with the results of Phillips et al. (2010) and our total molecular evidence analyses (Fig. 4a) because it includes a clade comprising all Australasian taxa whereas our analyses united the tinamous and moas. Because the moas were only represented by mitochondrial data, one could argue that the position of the moas is inaccurate. Although, even if the position of moas is inaccurate, the support for a rhea–Australasian clade is extremely limited, with none of the joint estimates of gene trees supporting this topology. The absence of support for this third topology is not consistent with the hypothesis that a hard polytomy provides the best explanation for the relationships among major groups of extant nonostrich paleognaths. Moreover, it is difficult to reconcile a set of estimated gene trees where only two of three possible trees predominate very strongly with a coalescent model, because that model predicts that the two less common gene trees would be equiprobable given a three taxon problem. Overall, the conclusions of this study and Harshman et al. (2008) suggest that substantially greater amounts of data (e.g., Faircloth et al. 2012) will be necessary to establish the sister group of the tinamous with confidence.

A Novel Emerging Paradigm for Paleognath Evolution

The independent evidence we obtained provided strong corroboration of the hypothesis that nonostrich paleognaths form a clade. This sharply alters our understanding of the evolutionary history of the flightless ratites by providing support for multiple losses of flight (for additional details see Harshman et al. 2008; Phillips et al. 2010). It remains possible there was a single loss of flight early in paleognath history followed by a regain of flight in tinamous, but this is unlikely because the loss of flight appears to be a relatively easy transition for birds (Feduccia 1996; Steadman 2006) whereas the loss followed by the regain of flight has never been documented. The hypothesis that flight has been lost multiple times in the ratites suggests that some of the most distinctive morphological characters in ratites arose through convergent evolution. Convergence

has been shown to mislead phylogenies (e.g., McCracken et al. 1999). Because the convergence postulated here is likely to be associated with postcranial anatomy, cranial characters may be less susceptible to convergence due to the loss of flight. Indeed, two morphological studies have described cranial characters that suggest nonostrich paleognath monophyly (Bock and Bühler 1990; Elzanowski 1995) and a third, more recent, morphological study presented a cladistic analysis congruent with our hypothesis (Johnston 2011).

The hypothesis of multiple losses of flight brings flexibility to biogeographic hypotheses of the group as well. The prevailing paradigm over recent decades has associated divergences among ratites with the break-up of Gondwana, the ancient supercontinent that gave rise to the large Southern landmasses. However, with the exception of the recent morphological phylogeny proposed by Johnston (2011), no proposed paleognath phylogeny is completely consistent with the geological history of Gondwana. Consideration of the mitochondrial sequence data available for the extinct moas further increases the inconsistency with geological history because it suggests two independent colonizations of New Zealand by ratites (Cooper et al. 1992, 2001; Haddrath and Baker 2001; Phillips et al. 2010). Although it may be possible for flightless birds to disperse by rafting or swimming, dispersal of volant ancestors followed by independent losses of flight may offer a more plausible explanation. The phylogenetic hypothesis that was corroborated by our independent evidence cannot exclude a role for Gondwana vicariance in paleognath biogeography, but it makes it unnecessary to postulate a direct relationship between ratite evolution and continental breakup.

The hypothesis of nonostrich paleognath monophyly also has the potential to explain the inconsistencies between the Gondwana breakup hypothesis and the ratite fossil record. The majority of paleognath fossils postdate the KT-boundary (Hope 2002; Parris and Hope 2002; Mayr 2009), inconsistent with the Gondwana breakup hypothesis. Indeed, Houde and Haubold (1987) pointed out that the "early Tertiary record of ratite birds is very poor, particularly in light of their large size which might be expected to improve their chances of being preserved." This emphasizes the surprising nature of the absence of ratite fossils expected given the Gondwana biogeography hypothesis. Moreover, a number of extinct northern hemisphere paleognaths have been described (e.g., Houde and Olson 1981; Houde 1986; Houde and Haubold 1987; Leonard et al. 2005; Mayr 2009), indicating that there must have been dispersal of paleognaths during the Paleogene or Cretaceous. Some of these extinct paleognaths (e.g., *Palaeotis weigelti* of the early Eocene of Europe) were flightless whereas others (the lithornids of the Eocene and Paleocene of Europe and North America) were volant. Houde (1988, pp. 109–113) presented a number of lines of evidence that lithornithids were strong flyers, suggesting that they may have been able to disperse long distances. This makes the hypothesis that the distribution of extant ratites reflects dispersal combined with multiple losses of flight plausible. In fact, it is likely that there may have been even more losses of flight than suggested by our phylogeny, especially when the fossil evidence for flightless paleognaths of the northern hemisphere is considered (e.g., Houde and Olson 1981; Houde 1986). Finally, we note that molecular clock analyses (e.g., Chojnowski et al. 2008; Phillips et al. 2010; Pyron 2010) suggest relatively recent divergence times that may be problematic for the Gondwana vicariance hypothesis. Taken as a whole, the hypothesis that nonostrich paleognaths form a clade represents a novel consilience that reflects the existence of a genome-wide phylogenetic signal in the nucleus (this work and Harshman et al. 2008), a congruent signal in the mitochondrial genome (Phillips et al. 2010), and the fossil record.

Conclusions

The independent evidence presented here strongly corroborates a paleognath phylogeny that includes a nonostrich paleognath clade (Fig. 1c). This independent confirmation was particularly important given the strong signal supporting ratite monophyly (Fig. 1a) in virtually all morphological and many molecular analyses. Evolutionary hypotheses suggested by phylogenies, particularly those that report novel conclusions, often stimulate other types of studies. For other venues of research to be productive, it is important that the phylogenies they are based on be rigorously tested. For example, now that multiple lines of evidence support a topology that indicates ratites have undergone multiple losses of flight, follow up studies to examine differences among ratites in their developmental processes might provide a fruitful way to examine morphological convergence. Likewise, comparisons of the biomechanics of movement in different paleognath clades may be worth exploring, as it is clear that the cursorial habits of the flightless taxa probably arose independently. Finally, tests of macroevolutionary hypotheses that use paleognath taxa (e.g., Laurin et al. 2012) can now be limited to topologies consistent with nonostrich paleognath monophyly. Other situations where long-standing and well-accepted relationships are overturned should also be rigorously vetted by generating independent evidence that can be used to test specific branches in the tree of life.

Supplementary Material

Data files and/or other supplementary information related to this paper have been deposited at Dryad under doi 10.5061/dryad.5vd2560f (www.datadryad.org).

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