AN INDEPENDENT TEST OF RATITE POLYPHYLY

By

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To my family and mentors

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The paleognaths are an ancient lineage of birds important for understanding early avian evolution, yet paleognath phylogeny remains unresolved, with the flightless ratites being most controversial. As some of the most well recognized of living birds, the ratites (modern paleognaths minus tinamous) include the giant birds of Africa, South America, Australia, and New Zealand. Although relationships within the ratites are unclear, the modern consensus on ratite phylogeny suggests they are the reciprocally monophyletic sister clade of the tinamous, volant paleognaths of the neotropics. In sharp conflict, a recent publication of the Avian Tree of Life Project supports ratite polyphyly because the ostrich emerges basal in the paleognath clade, nesting the volant tinamous within the flightless ratites. This study is an independent test of ratite phylogeny using a large nuclear dataset, further validating ratite polyphyly and the early divergence of ostrich from all other paleognaths. Phylogenetic artifacts, such as alignment bias and gene tree-species tree conflict, do not explain this result, nor does bias from locus selection strategies, specifically EPIC (Exon-primed Intron-crossing) and anonymous approaches. A species tree that supports paleognath monophyly but ratite polyphyly has significant implications for reinterpreting paleognath evolution.

CHAPTER 1
INTRODUCTION

Paleognaths are an ancient lineage of birds that emerge at the deepest branch in the avian tree of life (Groth and Barrowclough, 1999; Braun and Kimball, 2002; Livezey and Zusi, 2007; Hackett *et al.*, 2008; Harshman *et al.*, 2008). Although they comprise less then one percent of all avian species, paleognaths are some of the most widely recognizable living birds. Most distinct are the ratites, the giant cursorial birds of the southern hemisphere. The extant ostrich of Africa, the South American rheas, Australia's emu and cassowaries, and the kiwis of New Zealand all belong to this distinctive group. Also included among the ratites are the extinct moas of New Zealand and elephant birds of Madagascar. Initially recognized by their keel-less sternum, the ratites were first formally described in the early nineteenth century (Merrem, 1813). Closely related to the ratites are the tinamous of the neotropics. The ratites and the tinamous share unique palatal structures for which the paleognaths were named (Huxley, 1867). Unlike the ratites, the tinamous are volant. A unified paleognath clade with reciprocally monophyletic ratite and tinamou lineages was first proposed by Pycraft in 1900 (Fig. 1-1A).

Although the modern consensus on paleognath relationships broadly reflects that of Pycraft (1900), paleognath phylogeny has been in conflict throughout its long history. Neither monophyly of the paleognaths nor the ratites gained substantive support until the first avian cladistic works of Cracraft in 1974, more than a century later (see Sibley and Ahlquist (1990) for a thorough review). In early works, discrepancy was largely attributed to convergence, an idea supported by the disparate biogeography of the taxa (Parker, 1895; Furbringer, 1902; Mayr and Amadon, 1951; DeBeer, 1956). Over the last few decades, however, most morphological and molecular phylogenetic studies have supported unified ratite and paleognath

lineages, the exceptions including Houde and Olson (1981), Olson (1985), Houde (1986), Bock and Butler (1990), and Elzanowski (1995). Conflict has shifted largely to relationships within the ratites.

The recently published Avian Tree of Life presents another source of conflict in the evolutionary relationships of the paleognaths (Hackett *et al.,* 2008) (Fig. 1-1B). In sharp contrast with the modern consensus (Cracraft, 1974; Sibley and Ahlquist, 1990; Cooper *et al.,* 1992; Lee *et al.,* 1997; van Tuinen *et al.*, 1998, Cooper *et al.*, 2001; Haddrath and Baker, 2001; Livezey and Zusi, 2007), Hackett *et al.* (2008) maintain paleognath monophyly but reject ratite monophyly. Unlike previous studies, this large-scale nuclear DNA dataset confidently nests the volant tinamous within the flightless ratites, making the ratites polyphyletic. Using a substantially overlapping dataset, Harshman *et al.* (2008) examine this topology for phylogenetic artifacts. Bias from long-branch attraction, deviations in base composition, gene tree-species tree conflicts, and misguided alignments do not contribute to the controversial paleognath phylogeny presented in Hackett *et al.* (2008). Although preliminary, the results from Hackett *et al.* (2008) and Harshman *et al.* (2008) suggest ratite polyphyly is a robust, genome-wide signal.

Incongruence between the molecular datasets above and those supporting ratite monophyly (*e.g.*, Cooper *et al.,* 1992; Lee *et al.,* 1997; van Tuinen *et al.,* 1998; Cooper *et al.*, 2001; Haddrath and Baker, 2001) can be attributed to methodological bias such as lack of power (Qui *et al.,* 1999; Braun and Kimball, 2001; Chojnowski *et al.*, 2008), or improper analyses (Kimball and Braun, 2002; Phillips, 2004). A re-evaluation of mitochondrial data using more sophisticated models and increased taxon sampling recovers ratite polyphyly (Phillips *et al.; in press*). Importantly, this result indicates that the collective molecular signal may support the non-monophyly of ratites.

A topology supporting paleognath monophyly but ratite polyphyly sharply alters our understanding of the evolutionary history of the paleognaths. Considering the modern consensus, flightlessness among the paleognaths symbolizes the evolutionary divergence between the tinamous and the ratites; flightlessness is most parsimoniously explained by a single loss of flight in the lineage leading to the ratites (Fig. 1-2). The name ratite refers to the inability of ratite birds to fly and describes their raft-like sternum (Merrem, 1813). Under conditions of ratite polyphyly however, the assumption of a single loss of flight relatively early in paleognath ancestry is not fully supported. This scenario would have required a gain of flight in the tinamou lineage (Fig. 1-3A). Since there are no known instances of a regain of flight in birds, this is not a likely explanation. Instead, ratite polyphyly implies that flight was lost multiple independent times across at least three ratite lineages (see Harshman *et al*. 2008)(Fig. 1-3B). Multiple losses of flight have been well documented in over twenty avian lineages, most notably the rails (Feduccia, 1996; Steadman, 2006). Importantly, this alternative scenario would mean that flightlessness is not a synapomorphy of ratite birds and that some of the most distinguishable morphological characters in ratites arose through convergent evolution.

In this study, I provide an independent test of ratite polyphyly using a 40-gene dataset, doubling the amount of loci collected in Hackett *et al.* (2008) and Harshman *et al.* (2008). Over 22-kilobases (kb) of nuclear data were collected from across the genome, none of which have been used in any previous paleognath studies. To compliment and extend the analyses in Harshman *et al.* (2008), I examine the potential impact of phylogenetic artifacts due to gene tree-species tree incongruence (Degnan and Rosenberg, 2006) and alignment bias (Lake, 1991) using Bayesian and novel methods. I also investigate the potential bias of EPIC (Exon-primed Intron-crossing) (Palumbi and Baker, 1994) and anonymous locus selection strategies on phylogenetic

signal. To my knowledge, this is the first study to quantitatively compare these two approaches for deep avian phylogenetics.

One common strategy for locus selection is the EPIC approach (Palumbi and Baker, 1994). This approach utilizes the flanking coding regions for primer design of introns. Though convenient since primers are relatively easy to design in exons, the EPIC approach limits primer development to short introns. EPIC loci access less then 10% of the avian genome (estimate derived from Ellegren (2005)). In comparison, anonymous loci (Karl and Avise, 1993) are collected randomly from across the genome and are not constrained by exon/intron boundaries. Importantly, these loci represent arbitrary fragments of the genome accessing large introns and intergenic regions that are not available using the EPIC approach. I evaluate if phylogenetic signal is similar between EPIC and anonymous loci; a comparison that indicates whether the use of short introns, such as those used in Hackett *et al.* (2008) and Harshman *et al.* (2008), may mislead phylogenies.

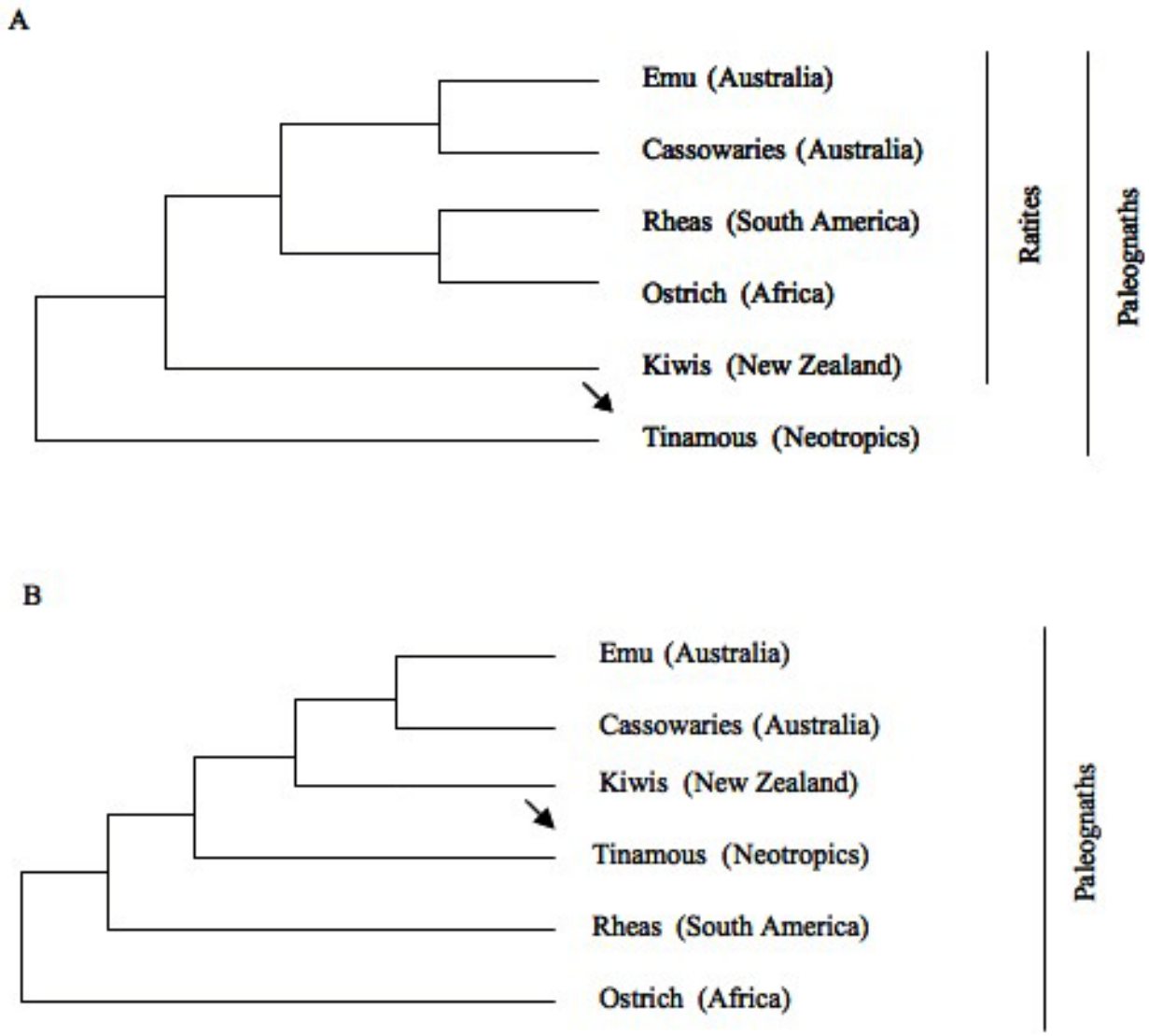

Figure 1-1. The modern consensus on paleognath phylogeny. A) supports monophyletic ratite and paleognath lineages (Cracraft 1974). In contrast, B) Hackett *et al.* (2008) maintain paleognath monophyly but support ratite polyphyly (both figures have been modified).

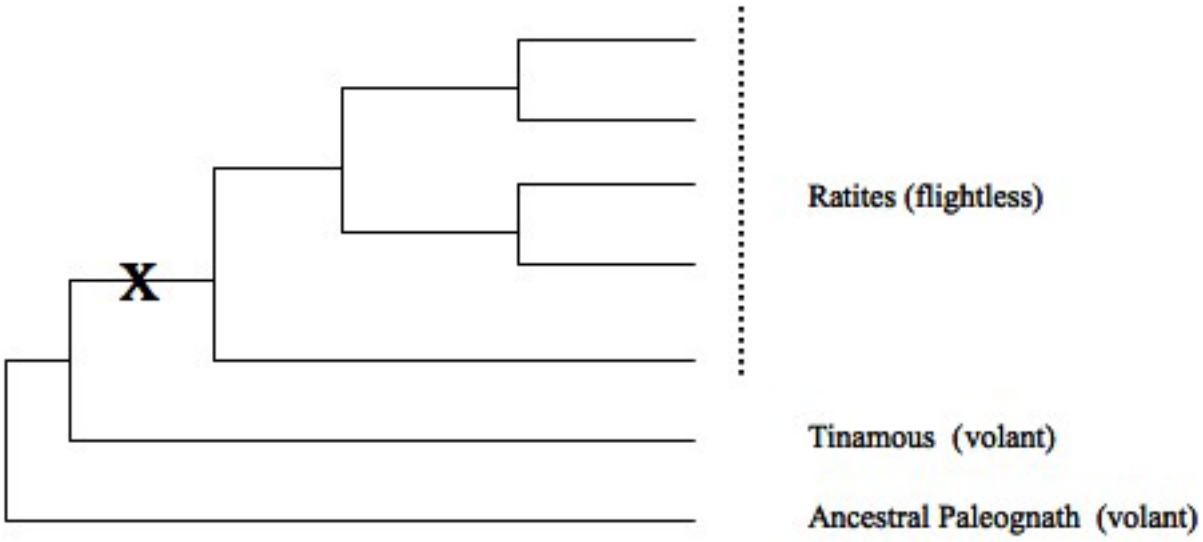

Figure 1-2. Patterns of flightlessness in the paleognaths when ratites are considered monophyletic and the ancestral paleognath is assumed to be volant. Patterns of flightlessness among the paleognaths can be most parsimonious explained by a single loss of flight as indicated by the X on the tree. This hypothesis suggests that flight was lost relatively early in paleognath evolution.

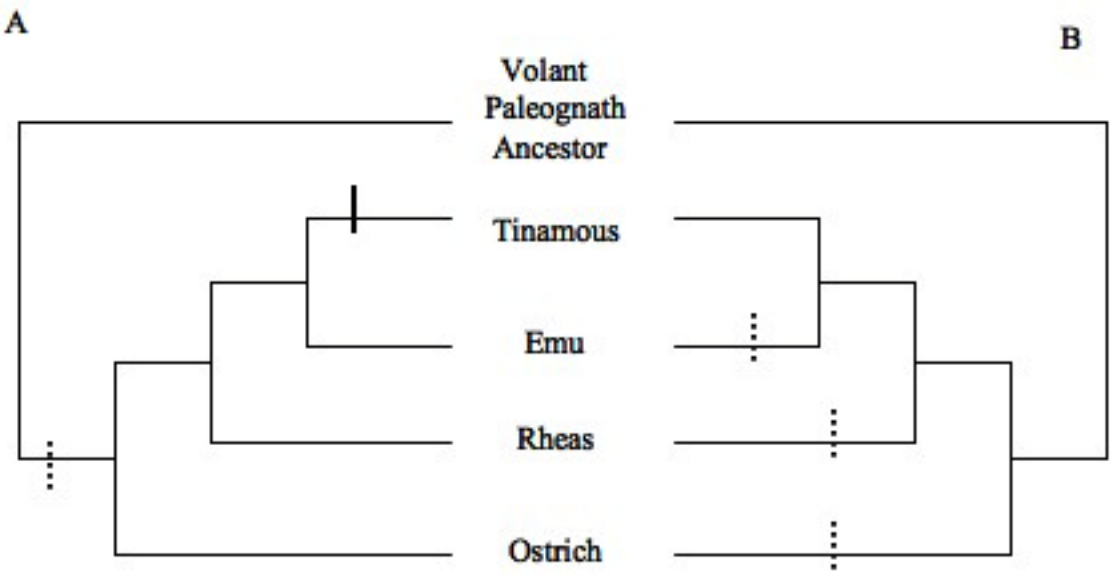

Figure 1-3. Patterns of flightlessness in the paleognaths when ratites are considered polyphyletic and the ancestral paleognath is assumed to be volant. Dashed lines represent loss of flight, the solid line represents a gain of flight. Since there are no know instances of flight being regained in birds A), multiple independent losses of flight are considered the most likely hypothesis B). Multiple Independent losses of flight suggest that some of the most distinguishable ratite characters may exhibit homoplasy.

CHAPTER 2
METHODS

Taxa

Ten taxa are sampled in this study (Table 2-1) including two tinamous: *Crypturellus soui* (Little Tinamou) and *Tinamus guttatus* (White-throated Tinamou). The ratites are represented by the rheas, *Pterocnemia pennata* (Lesser Rhea) and *Rhea americana* (Greater Rhea) as well as *Struthio camelus* (Ostrich). Since the Australasian clade is strongly united in Harshman *et al.* (2008) only one Australasian taxa was used in this study, *Dromaius novaehollandiae* (Emu). In addition, three representatives of Galloansere were used as outgroups: *Chauna semitorquata* (Southern Screamer), *Crax alector* (Black Currasow), and *Gallus gallus* (Chicken). Providing a neoaves representative, *Taeniopygia guttata* (Zebra Finch) was included in the dataset using sequences available from NCBI traces archives.

Locus Development

Two locus selection strategies were used to generate this data: the anonymous approach and the EPIC (exon-primed intron-crossing) approach. In total, 10 anonymous loci were used for phylogenetic analyses (Table 2-2). 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. Genomic DNA was sheared via sonication to produce fragments in the range of 2-kb. 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). Library Clones were selected randomly and sequenced as described below. Candidate regions for anonymous primer design were identified by comparing sequence

similarity between chicken and the tinamou sequences with BLASTN searches. Primers were designed from non-repetitive, homologous regions greater than 300 bases in length.

To provide comparison to the anonymous loci, 30 EPIC loci were also included in the dataset. Twelve EPIC loci were from previous non-paleognath studies (Table 2-2) and the remaining 18 were developed for use in this study. EPIC loci were designed using the general philosophy described by Kimball *et al.* (2009). Chicken was compared against Zebra Finch ESTs (Express Sequence Tags) and other available avian data for primer design. Homology was assessed using chicken BLAT (Kent, 2002). BLAT was useful because it distinguishes exon boundaries facilitating primer design in the coding regions. Primers were designed to isolate short introns (averaging 500 bp) that do not require internal primers.

Amplification and Sequencing

PCR amplification (Polymerase Chain Reaction) was achieved through standard procedures. Products were cleaned by precipitation using an equal volume of PEG:NaCl (20 %:2.5M). Several primer sets required purification via gel electrophoresis using the Perfectprep®Gel Cleanup (Eppendorf). Cycle sequencing was performed using ABI BigDye® Terminator v.3.1 and sequences were obtained using an ABI Prism™ 3100-Avant genetic analyzer (PE Applied Biosystems). If length polymorphisms between alleles resulted in unusable sequence data, these PCR products were cloned using the pGEM®-T Easy vector (Promega Corp.). Plasmids were prepared for sequencing using the Perfectprep® Plasmid Mini kit (Eppendorf) and sequenced using the same protocol I used for PCR products. Sequences were examined and assembled into double-stranded contigs using Sequencher™ 4.1 (Gene Codes Corp.).

Alignment

Two alignment strategies were used in this study. First, contigs were imported into MacClade 4.0 (Maddison and Maddison, 2000) and alignments were optimized by eye. These were the primary alignments used in the phylogenetic analyses and locus characterization. Second, a progressive alignment program, the Probabilistic Alignment Kit (PRANK), was used to generate automated alignments (Loytynoja and Goldman, 2005). The PRANK alignments were used to evaluate alignment bias in phylogenetic signal.

Alignment bias was induced in PRANK by manipulating the guide trees used in alignment construction. When the tinamous, rheas, and outgroups were constrained as monophyletic lineages, there were only 15 possible trees. All 15 topologies were used as guides to generate bias in 15 sets of corresponding alignments. Maximum likelihood (ML) analyses of the 15 sets of alignments were then conducted in PAUP* 4.0b10 (Swofford, 2003) using random addition heuristic searches. Support was assessed using 1000 bootstrap replicates. Topologies of the 15 biased datasets were then compared to determine if alignment bias influenced phylogenetic signal.

Phylogenetic Analyses

Multiple analyses were conducted on each individual locus as well as three separate concatenated datasets: an anonymous locus partition, an EPIC locus partition, and the total combined dataset. Maximum parsimony (MP) and ML analyses of all 40 individual gene trees were performed using PAUP* 4.0b10 using branch and bound searches. ML and MP analyses of the three concatenated datasets were conducted in PAUP* 4.0b10 (Swofford, 2003) using 10 random addition heuristic searches with TBR branch swapping. For ML analyses, the appropriate model for each partition was determined by the akaike information criterion (AIC) in MODELTEST 3.06 (Posada and Crandall, 1998). Support for all MP and ML analyses was

examined by 1000 bootstrap replicates using heuristic searches. A topology test using the Shimodaira- Hasegawa test in PAUP* 4.0b10 was conducted on all 15 possible topologies to further evaluate the best likelihood tree topology from the total, combined dataset (Shimodaira and Hasegawa, 1999).

In addition to the concatenated ML analyses in PAUP, partitioned ML analyses were conducted using RAxML (Stamatakis, 2006) (Table 4). For each separate concatenated dataset, data was partitioned by locus and searches used a GTRMIX model. Also, partitioned bayesian analyses of the three concatenated datasets were conducted using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Here the appropriate model for each partition was determined using the AIC after limiting the models to those implemented in MrBayes. Each analysis consisted of two simultaneous searches of 50 million generations with burn in values of 5 million generations. Convergence was assumed when the average standard deviation of the split frequencies for the simultaneous runs approached zero, the potential scale reduction factors (PSRF) approached one, and the likelihood values reached stationary values.

In this study, the majority of gene trees supported the basal divergence of ostrich however, the most frequent gene tree might not represent the true species tree in some instances (Edwards *et al.*, 2005; Degnan and Rosenburg 2006; Kubatko and Degnan, 2007). To examine if gene tree-species tree discordance influenced my results, I used Bayesian estimation of species trees (BEST) (Liu and Pearl 2007). BEST generates a species tree without concatenation of the loci by accounting for gene tree variability. As mentioned for the MrBayes runs above, the complete concatenated dataset was modeled by locus and two simultaneous runs were conducted for 50 million generations. Burn in was set at 5 million generations. Chicken and Zebra Finch were chosen as outgroups among the four neognath taxa and were evaluated in separate analyses.

Locus Characterization

Estimates of stationary base composition and evolutionary rate for locus characterization were calculated using PAUP*4.0b10. Evolutionary rates were based on the ML trees and were normalized using the rate of the total evidence tree. The position of each locus on the chicken chromosome and the corresponding gene association was determined using the NCBI database (Table2-3).

Table 2-1. Sampled Specimens

	Species	Common name	Instituition*	Voucher or tissue number
Paleognaths				
	Dromaius novaehollandiae	Emu	LSUMNS	B5895
	Rhea americana	Greater Rhea	FLMNH	44923
	Pterocnemia pennata	Darwin's Rhea	USNM	620827
	*Struthio camelus***	Common Ostrich	LSUMNS	B1526
	Crypturellus soui	Little Tinamou	USNM	586295
	Tinamus guttatus	White-throated Tinamou	FMNH	389673
Galloanseres				
	Crax alector	Black Curassow	USNM	625104
	Chauna torquata	Southern Screamer	USNM	614546

* Institutions who provided the specimens were: FLMNH, Florida Museum of Natrual History, FMNH, Field Museum of Natural History, LSUMNS, Louisiana State University Museum of Natural Science, USNM, United States National. ** The *Struthio camelus* DNA used for this study was not formally vouchered but was validated using loci from the provided specimen and published in Hackett *et al*. (2008).

Table 2-2. Primer Information

Locus	Forward Primer	Reverse Primer	Gene Association
Anon1	CTGCTAAAWAGAATCCNGG	GRAGTCATTRTGCACCCTC	SLC25A21
Anon2	GGATGCTCGCTCAGKAMTTTG	GTGGTTTAGCCTGGAGTTAAG	--
Anon3	CTGCACWGGAAGGCTKATG	GTCTGAWGATGTCTSAGCTGTG	PALLID
	TCCTGGYCAGCCTCATRTTAGAAG		
Anon4	GTCCACCAGTNMGGTATTAATC	CCTNCCACTGTANCAAANG	BMP5
	GAGGGGAAACTTGGAAYTTTGTGG	GAGGGGAAACTTGGAAYTTTGTGG	
Anon5	GGAGACCTTRTTGGACATATTGTTG	CACMGCTTCAATGAGACACCTC	PUM2
Anon6	GCTGCACTYATAGKTRAGG	GACCAACTGAAAAGACTTG	RXFP2
	AATGTGGGACTYAAGGAAGCTG	CAAYAGCCAAGGTCCTGATTCAG	
Anon7	GGYTGTTGAGAGRACAGAAGG	GTTTKGGCAGGTRCTGGC	NUSAP1
Anon8	CACAACACTTGACTATGGC	GAYKCAGACACCRCATTATC	--
Anon9	GGCAGYATTAAGGAAACGCAC	GAGTAYGTAGACCAAMCCATACG	TTN
Anon10	GTTATCAAGTGATCTGTTTGCAGTC	GCAKCYGTGATGCCAGGRTG	--
Epic1	GGTTGGAGAACTTGTTTATGG	GGCTCATAAAGGGCTTTG	GRIA2
Epic2[3]	CCTGATGGTCAGGTCATCA	CAGCAATGCCAGGGTACAT	ACTB
Epic3[1]	TGGTTCAGTTTCATGAACCCTTG	CCTGAAGCACRCTGTCCATGCT	ARNTL
Epic4[2]	AGGGTGTCAARATGTGTGSGAAAGA	GTANAGCTTCCCTCCATCNGACAA	CALB1
Epic5	AGAAAGGCCTGGAGAGGAGAGC	GTCTTCAACCACCAGTCCGAGAG	CRAT
		CATGSTATCTGCTGCTGTTYGGTCC	
Epic6	GACTACGTCTTTGACTGGAACATGCT	ATCCTCAGGGTTTCGGGCTG	CSNK1E
Epic7	CCCTGAATCAGCCCTCAAATTCTACTGTTA	AATCTCCCCAAGTCGCTGCTG	CIZ1
Epic8[1]	CCAGAGGGGGAACATTCAGAA	TCCTTTGGGTCTATTGTTCCTCG	CLOCK
	CATGTGGATGATCTAGATAATCTGGC*	GYAATGTGTTTGCAGCCAAATCCA*	
Epic9[1]	CTGGTGCTGTAAGTGCTCGTAAC	CCAGGCTGTAAGGTTTCTAGGTCAC	CSDE1
Epic10	CATATAAATCATCAGCCATTCCTGG	GTTGGTGCCAGCACAAGAC	DDX5
Epic11[1]	GCAGGCCTRRCTGGAAAAGARCC	TTCTGAGCTCCWAGRTTACC	PARK7
Epic12	GTCCAGCAATGAGACACCTCCAC	CCAGTCATCATCGTCCTCCTCC	EIF5
Epic13	GGTGATGATCTGACTGTGACCAACC	CATCACACCCCAGCCATTGGAC	ENO1
Epic14	CAGTGGCTTCACAAAGGAACAGTGTC	CAAACATGCTGTTCAGTCCACAACC	ETS2
Epic15[2]	TGCGGGTGCTGGCATTGC	TGCATGCCATGTGGACCAT	GAPDH
Epic16	GATCACCTCCTGCTTCAGCTC	GGCCCCAGCATCAAGATCTG	GNB2L1
Epic17	GCATTTCCTGTCTAGAGAGGGCTTTC	CATTTGATGACCATGATCCTGTGTGG	HNRPA2B1
Epic18	CTAAGTAGGAATTGTCTTCATCAGC	GATGAAGACGATTTGGAAGC	CHMP5
Epic19	CATGGACCGAAGAGGAGGCACT	CCAGAGAGCATCTGCATATGTGGAG	KCNQ5
Epic20[1]	CCCTCAGACACTGGATTAYGAATCAT	CCAAGGATTCCGAAGCAGTAAG	PAXIP1
Epic21[1]	ATCAGAGGGGTTCTCAAAGATGG	AGAGAAGGCTCTGGGCTTGTCGGTA	NAT15
Epic22[1]	CATCTTCAYCCAAATGACAGACC	CCTGATTGGTGAATAGTCAAAAGG	PER2
Epic23	TGGGGYTGGCTGTNGCRGGTGGAGT	CAGGGGATGAGGAAGTGGGTRCCTTC	PHB
Epic24	GTATAGTGGTATGGGTCCAGATTAC	GCTGTTGGAATGGGCTCATGATAAAC	PSMA2
Epic25[1]	GGAAACCCCAGCTACAAGTATTTC	GGCCTCCTTCATCCCTTGG	TXNDC12
Epic26	GCTGTGTATTTGGTCTATTCAGAG	CAGGTGGCAAATGTAAAGATGTG	SFRS3
Epic27	CTTGGCGATCACAGGGACAATG	GAACAGGCGCCACATTATAGACAATAG	SEPT2
Epic28	CCCNGATCGCAAAAATCTGAAATG	CGAAGAATAGTAATTGCWGCTTCTGTTGC	TCP1
Epic29	GTCAACGTCACAACCTAGG	CAACTTTCTCACCAAATACAGG	VDAC2
Epic30[1]	GACCGTGGAAACTAGAGATGGAC	GTCATCGTGATGCTGGGAAGTTTC	VIM

[1]Loci can be found in Kimball *et al.* (2009), [2]loci in Cox *et al.* (2007), and [3]loci in Waltari and Edwards (2002).

Table 2-3. Locus Characterization

Locus	Gene Association	Chromosome*	Base Composition Stationary	Normalized rate**	ML Support For Ratite Polyphyly***
Anon1[a]	SLC25A21	5	yes	0.78	yes
Anon2[b]	--	2	yes	0.98	yes
Anon3[d]	PALLID	4	NO	0.91	yes
Anon4[a]	BMP5	3	yes	0.18	NO
Anon5[d]	PUM2	3	yes	0.85	yes
Anon6[a]	RXFP2	4	NO	1.44	NO
Anon7[d]	NUSAP1	5	yes	0.95	NO
Anon8[b]	--	Z	NO	0.96	yes
Anon9[c]	TTN	7	yes	0.25	yes
Anon10[b]	--	8	yes	0.97	yes
Epic1	GRIA2	4	yes	0.86	yes
Epic2	ACTB	10	yes	1.34	yes
Epic3	ARNTL	5	yes	0.98	yes
Epic4	CALB1	2	yes	0.70	yes
Epic5	CRAT	17	NO	0.84	yes
Epic6	CSNK1E	2	yes	1.07	yes
Epic7	CIZ1	17	yes	0.63	yes
Epic8	CLOCK	4	NO	1.01	yes
Epic9	CSDE1	2	yes	1.10	yes
Epic10	DDX5	18	yes	1.59	yes
Epic11	PARK7	21	yes	1.00	yes
Epic12	EIF5	5	yes	1.76	yes
Epic13	ENO1	21	yes	1.37	yes
Epic14	ETS2	1	yes	0.57	yes
Epic15	GAPDH	1	yes	1.33	NO
Epic16	GNB2L1	16	yes	2.20	yes
Epic17	HNRPA2B1	2	yes	1.47	NO
Epic18	CHMP5	2	yes	1.26	yes
Epic19	KCNQ5	3	yes	0.86	yes
Epic20	PAXIP1	2	yes	0.95	yes
Epic21	NAT15	14	yes	1.22	yes
Epic22	PER2	9	yes	1.27	yes
Epic23	PHB	27	yes	1.26	yes
Epic24	PSMA2	2	yes	1.11	yes
Epic25	TXNDC12	8	yes	0.90	yes
Epic26	SFRS3	z	NO	0.91	yes
Epic27	SEPT2	15	yes	0.95	yes
Epic28	TCP1	3	yes	1.55	yes
Epic29	VDAC2	6	yes	1.18	yes
Epic30	VIM	2	yes	1.24	yes

Anonymous locus types are represented by [a] large intron, [b] intergenetic region, [c] exon only, and [d] intron plus exon. * Chromosomal position of the locus is referenced to the chicken genome. ** Individual locus rates were normalized by the total evidence rates. *** Support for ratite polyphyly is shown based on the single best ML topology for each locus.

CHAPTER 3
RESULTS

Concatenated Trees

This 40-locus dataset provided independent support that the ratites are not monophyletic. In all combined analyses, non-ostrich paleognaths are united at the basal node of the tree nesting tinamous within the ratites and supporting the early divergence of the ostrich (Fig. 3-1). Although controversial, this critical node was supported by a bootstrap value of 100 in nearly all maximum parsimony (MP) and maximum likelihood (ML) analyses, and a posterior probability of 1 in all Bayesian analyses (Table 3-1). Furthermore, the basal emergence of ostrich is congruent with Harshman *et al.* (2008) and Hackett *et al*. (2008) and provides strong support for ratite polyphyly using nuclear DNA.

Although the basal position of the ostrich is well resolved, the specific relationship of the tinamous within the non-ostrich ratites remains uncertain (Table 3-1). An SH test reveals that none of the three possible sister relationships of the tinamous with the non-ostrich ratites can be rejected as significantly worse hypotheses (Table 3-2). Although kiwis are not represented in this dataset, including this lineage would likely not have improved resolution of the tinamou sister clade; the phylogenetic position of the tinamous was unclear in Harshman *et al.* (2008) despite inclusion of the kiwi lineage. A combination of small taxon number and variable internodes lengths makes this a particularly difficult phylogenetic problem (Lee *et al*., 1997).

Individual Gene Trees

Ultimately, 35 of the 40 loci supported ratite polyphyly using ML analyses (Table 3-3). Nine of these loci supported a non-ostrich ratite emerging at the basal node and the large majority, 26 loci, supported the basal position of the ostrich. Interestingly, the basal emergence of ostrich is supported by 65% percent of the total gene trees, a similar proportion as found in

Harshmen *et al.* (2008). For the four gene trees that supported ratite monophyly, the evolutionary rate is relatively fast, although similar rates occur for gene trees supporting ratite polyphyly. The results of the MP analyses were similar. Only eight loci supported ratite monophyly and the large majority of trees (32) supported ratite polyphyly (Table 3-3).

Gene Tree-Species Tree Incongruence

For this dataset, Bayesian estimation of species trees (BEST) supports a species tree with ratite polyphyly and has ostrich emerging at the basal node regardless of which outgroup was used for analysis (Fig. 3-2). This result confirms the ML, MP, and Bayesian topologies and suggests that gene tree-species tree discordance is not a strong source of discrepancy between this large-scale molecular study and those of previous phylogenetic studies.

Alignment Bias

No matter which of the 15 guide trees was used to generate alignments in PRANK, each resulting concatenated dataset supported ratite polyphyly. Furthermore, ostrich emerged at the critical node with ML bootstrap values of 100 in all 15 topologies. Therefore, even when the guide tree biased alignments toward ratite monophyly, the phylogenetic signal for ratite polyphyly was recovered with confidence. Alignment bias did not affect the critical node in this alternative topology (data not shown).

There was some evidence for alignment bias in determining the sister taxa of the tinamous. For example, when the three possible topologies supporting ratite polyphyly with ostrich basal were used as guides, two alternative sister relationships had conflicting bootstrap values of 99 or greater (Table 3-4). This demonstrates the potential for alignment bias when the phylogenetic signal is weak therefore, I advocate cautious use of PRANK when phylogenetic

relationships are uncertain. Other programs that do not incorporate models of substitution in the alignment process may be less susceptible to guide-tree bias (for review see Nelesen *et al.*, 2008).

Bias in Locus Selection Strategy

Both anonymous and EPIC (Exon-primed Intron-crossing) partitions clearly supported the basal position of ostrich among the paleognaths (Table 3-1). Therefore, ratite polyphyly cannot be attributed to locus selection bias. As might be expected, the placement of the tinamous is unclear between the two. Ultimately, short introns isolated by the EPIC approach and anonymous loci accessing long introns and intergenic regions seem to recover similar phylogenetic signal.

Table 3-1. A summary of the core phylogenetic analyses for the three concatenated datasets. For all analyses support uniting a non-ostrich paleognath clade and therefore ratite polyphyly is 100% except in two cases (*). The support values shown correspond to the tinamou sister relationship for the total concatenated dataset (T), the anonymous partition (A), and the epic partition (E).

Topology	Partitioned Mr.Bayes	Partioned ML	Concatenated ML	Concatenated MP
(Ostrich, (Emu,(Rheas, Tinamous)))		589-T, 641-E		61-T, 62-E
(Ostrich, (Rheas,(Emu,Tinamous)))	97-T, 91-E	422- A*	72-T, 62-E	37-A*
(Ostrich, (Tinamous,(Rheas,Emu)))	71-A		70-A	

* Support uniting a non-ostrich paleognath clade is 100 for all analyses except the anonymous MP and partitioned ML analyses that are 90 and 97.5% respectively.

Table 3-2. P-values from a Shimodaira-Hasegawa (SH) test. An SH test compared the most likely tree topology from the concatenated ML analysis of the complete dataset to all 15 possible tree topologies. All topologies with no significant difference in likelihood values from the best topology are shown.

Topology	P-values
(Ostrich, (Emu,(Rheas, Tinamous)))	0.725
(Ostrich, (Rheas,(Emu,Tinamous)))	Best topology
(Ostrich, (Tinamous,(Rheas,Emu)))	0.735

Table 3-3. Bootstrap support for ratite polyphyly at the critical node uniting all non-ostrich paleognaths from maximum likelihood and maximum parsimony analyses

	ML		MP	
Locus	Support	Conflict	Support	Conflict
Anon1	--	72	--	52
Anon2	94	--	89	--
Anon3	84	--	85	--
Anon4	--	25*	--	--
Anon5	73	--	49	--
Anon6	--	60*	--	93*
Anon7	--	--	--	--
Anon8	83	--	90	--
Anon9	46	--	64	--
Anon10	69	--	69	--
Epic1	43	--	--	--
Epic2	100	--	89	--
Epic3	86	--	88	--
Epic4	69	--	--	--
Epic5	99	--	88	--
Epic6	66	--	--	81*
Epic7	53	--	38	--
Epic8	72	--	74	--
Epic9	72	--	54	--
Epic10	--	50	--	--
Epic11	90	--	89	--
Epic12	--	40	--	42
Epic13	74	--	76	--
Epic14	86	--	82	--
Epic15	--	50*	--	75*
Epic16	68	--	48	--
Epic17	--	45*	--	53*
Epic18	--	42	--	48
Epic19	68	--	--	--
Epic20	--	65	--	61
Epic21	--	24	--	69
Epic22	67	--	--	45*
Epic23	--	82	--	36
Epic24	45	--	--	50*
Epic25	80	--	86	--
Epic26	96	--	93	--
Epic27	73	--	89	--
Epic28	--	37	--	34*
Epic29	--	73	--	69*
Epic30	52	--	57	--

* Indicates loci which supported ratite monophyly. If multiple best trees conflicted at the critical node, results are not presented for that locus.

Table 3-4. Resulting maximum likelihood topologies using guided alignments biased for ostrich basal and ratite polyphyly.

Guide Tree	ML Bootstrap Support	
	Tinamous + Rhea	Tinamous + Emu
(Ostrich, (Emu,(Rheas, Tinamous)))	99	
(Ostrich, (Rheas,(Emu,Tinamous)))		99
(Ostrich, (Tinamous,(Rheas,Emu)))	84	

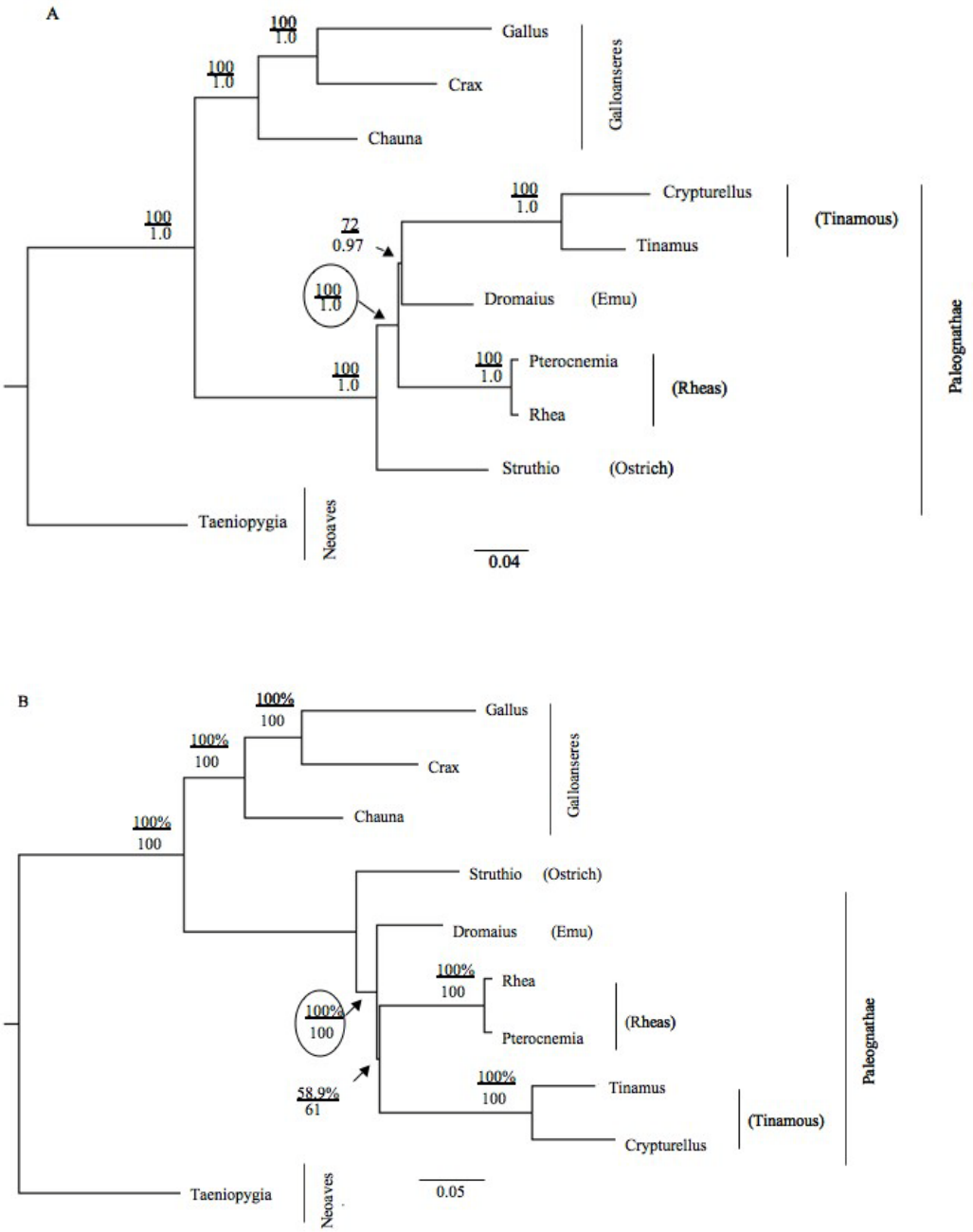

Figure 3-1. Multiple phylogenetic analyses indicated ratite polyphyly. Using 22kb of nuclear data, the topology from the concatenated ML analysis and the partitioned Bayesian analysis of the complete dataset is shown in A). Branch lengths are represented from the concatenated ML analysis. For each node, the top number represents the bootstrap value and the bottom number represents the posterior probability. The topology from the partitioned ML analysis and the un-partitioned MP analysis of the complete dataset is shown in B). Support values for the partitioned ML analysis are on top and un-partitioned MP bootstrap values are on bottom. Branch lengths are represented from the partitioned ML analysis. The critical node indicating ratite polyphyly is circled on both topologies.

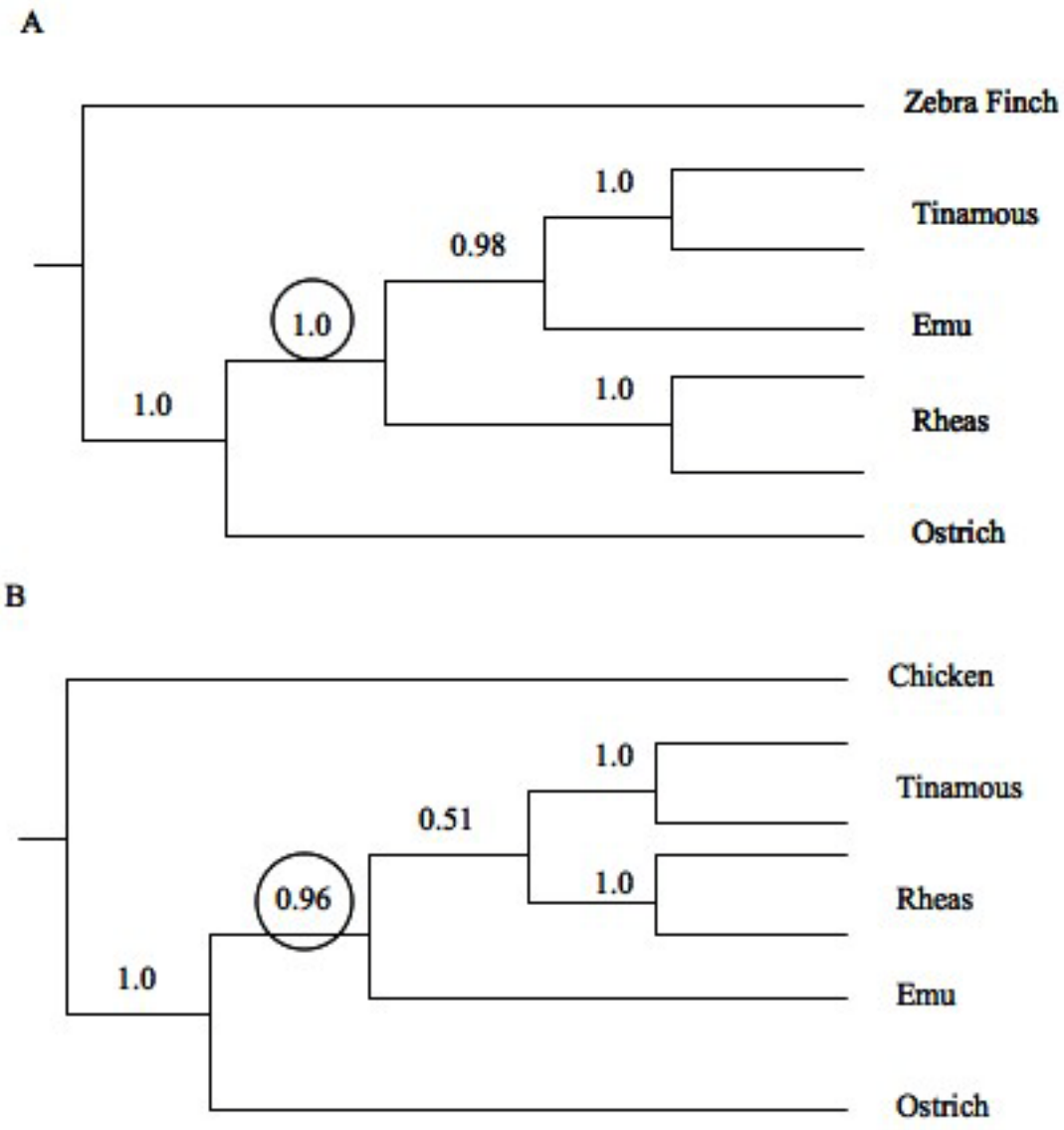

Figure 3-2. The paleognath species tree supported ratite polyphyly. BEST analyses of the complete dataset using A) zebra finch and B) chicken as neognath outgroups have tinamous nested within the ratites. Support values are shown and the critical node indicating ratite polyphyly is circled.

CHAPTER 4
DISCUSSION

An Independent Test of Ratite Polyphyly

This independent dataset provides strong and consistent support for ratite polyphyly through a variety of analyses of nuclear DNA. Phylogenetic signal was robust to alignment bias at the critical node, and neither locus selection strategy or gene tree-species tree conflicts appear to have misled analyses. My results support the novel conclusions of Hackett *et al.* (2008) and Harshman *et al.* (2008).

Evolutionary Implications

A species tree that supports ratite polyphyly has major implications for paleognath evolution. As mentioned previously, multiple independent losses of flight may mean that some of the most distinguishable ratite characters arose through convergent evolution such as reduction of the sternal keel, reduction in wing structure, opening of the pelvic region, emphasis of hind-limb structure, and larger size (Feduccia, 1996). Convergence has been shown to misled phylogenies (McCracken *et al.* 1999) and the possibility of convergence among the ratites is alarming. Not only does this hypothesis call to question previous synapomorphies of the clade, as suggested by Harshman *et al.* (2008), it offers an unambiguous explanation for the discrepancy between molecular and morphological phylogenetic signals. Two morphological studies using cranial characters further validate this hypothesis (Bock and Buhler, 1990; Elzanowski, 1995). Cranial characters have been proposed as being less susceptible to convergence from loss of flight (Bock and Buhler, 1990; Feduccia, 1996) and both studies support ratite polyphyly.

Biogeographic Implications

Ratite polyphyly offers some flexibility for resolving paleognath biogeography, a question that has long fascinated researchers. The distribution of these flightless birds has previously been related to vicariant events under the assumption that ancestral ratites were flightless (Cracraft ,1974; Sibley and Ahlquist, 1990). Given their broad distribution across the southern hemisphere, their divergence has been attributed to continental drift and the break-up of Gondwana (Cracraft, 1974). However, recent mitochondrial studies suggest dispersal must be considered (van Tuinen *et al*., 1998; Haddrath and Baker, 2001; Cooper *et al.,* 2001). Two studies were able to incorporate data from the extinct moas of New Zealand and found that the two New Zealand lineages (the moas and the kiwis) were not each other's closest relatives (Haddrath and Baker, 2001; Cooper *et al.,* 2001). This result suggests there were two independent colonization events of New Zealand with only the earliest divergence (the moas) possibly coinciding with the split of New Zealand from Antarctica. Although flightless birds have the ability to disperse by rafting or swimming (Cooper *et al.,* 1992), if ancestral paleognaths were prevalently volant as implied by ratite polyphyly, flight offers a more logical mechanism for explaining paleognath biogeography.

Good dispersal capabilities would no longer constrain ancestral paleognaths to a Gondwanan origin. As indicated by the fossil record, ratites and paleognaths did have a distribution in the Northern Hemisphere (Houde, 1986, 1988; Houde and Haubold, 1987; Mayr, 2005). Several ratite fossils have been identified in Europe. The most well known is *Palaeotis weigelti* of the early Eocene (Houde and Haubold, 1987). *Palaeotis* has a close affinity to either the ostrich (Houde and Haubold, 1987) or the rheas (Peters, 1992b). *Remiornis minor*, the oldest presumed ratite of the northern hemisphere, coincides closely in geologic time with the earliest ratite known in the southern hemisphere, *Diogenornis fragilis* (Alvarenga and Olson, 1983).

Although the precise affinity of *Remiornis* is debated, it suggests the possible simultaneous distribution of ratite-like paleognaths in the northern and southern hemisphere in the late Paleocene (Houde and Haubold, 1987; Feduccia, 1996). Considering ambiguity in the fossil record and the possibility for flight, a Laurasian origin for the paleognath clade should not be dismissed.

Subsequently, I suggest a re-examination of the extinct lithornithids and their place on the paleognath tree. Lithornithids are volant fossil paleognaths found in North America and Europe during the Paleocene and Eocene (Mayr, 2005). They have never been examined in a rigorous phylogenetic context although, they are generally considered to be ancestral tinamous (Houde and Olson, 1981; Houde, 1988; Feduccia, 1996; Leonard *et al*., 2005). If paleognath ancestors were prevalently volant, then the lithornithids may have a more central role in paleognath evolution as suggested by Feduccia (1996) and consistent with the predictions of Parkes and Clark (1966). Remarkably, a recently identified fossil of *Lithornis* has a well-preserved skull that may allow for the phylogenetic assessment of the lineage while avoiding the potentially convergent characters of the postcranial skeleton (Leonard *et al.,* 2005).

Although a species tree that supports ratite polyphyly clearly broadens biogeographic hypotheses for the clade, it is important to note that this phylogeny does not refute all aspects of a Gondwanan-based vicariance model. Ostrich emerges basal in the tree possibly corresponding to the initial split of Africa from Gondwana (Szatmari and Milani, 1999). Ultimately, resolution of paleognath biogeography will require good estimates of molecular divergence for direct comparison to the geologic time frame.

Anonymous Primer Utility

Despite the overall consensus in paleognath topology between the anonymous and EPIC loci, we recommend careful use of anonymous loci for multi-locus studies of deep phylogenetic questions. We found that the EPIC (Exon-primed Intron-crossing) approach yielded over three times as many phylogenetically informative markers than the anonymous approach when spanning a relatively large evolutionary depth. In my study, phylogenetically informative markers are defined by the non-paralogous locus amplification of all critical taxa: the ostrich, one tinamou, and at least one other non-ostrich ratite. According to mitochondrial estimates, the paleognaths last shared a common ancestor roughly 90 million years ago (Haddrath and Baker, 2001). Here, the proportion of phylogenetically useful anonymous loci was only 23% of the 43 initially examined. In comparison, 50% of the 60 EPIC loci were phylogenetically useful. In birds this suggests that the anonymous approach is less efficient than the EPIC approach for resolving deep phylogenies.

When isolating genomic regions across inter-specific taxa, anonymous loci are more slowly evolving then EPIC loci on average (Table 2-3). This is most likely a constraint due to methodology specifically from homology requirements for primer design rather then an intrinsic property. Anonymous loci have an average rate of 0.83 substitutions per site and EPIC have an average rate of 1.15 substitutions per site. The two most slowly evolving regions of the entire dataset are anonymous loci. The slowest is Anon4, a large, conserved noncoding region of the developmental gene BMP5. This is congruent with other studies suggesting that introns of developmental genes should be avoided when variability is needed (Woolfe *et al*., 2005). Surprisingly, the rate of evolution of Anon4 is even slower then Anon9, a protein coding region.

Another potential downfall of the anonymous locus approach is having no control over the physical location of the sampled loci. Although in some instances this may be viewed as an advantage, the macro-chromosomes are overrepresented. In addition, anonymous loci more frequently deviate from stationary base composition meaning they more frequently violate the evolutionary models currently used in phylogenetic analyses. There are no distinguishing patterns in locus characteristics between the four anonymous locus types (Table 2-3).

Anonymous primers have been most commonly used to address population level questions, typically as an alternative to microsatellite data (Hare *et al.*, 1996; Kuhner *et al*., 2000; Jennings and Edwards, 2005). To my knowledge, this is one of only two studies (Thomson *et al*., 2008) to examine anonymous primer utility for deep phylogenetic questions. Analysis of both studies suggests a trend of decreasing anonymous efficiency over large depths. First, considering efficiency of the anonymous approach in the paleognaths, only 3.28% of all the anonymous regions examined resulted in phylogenetically useful loci. That represents only 10 out of the original 304 library clones that produced readable sequences. Only 14.14% (43) of the readable sequences yielded candidate loci (as defined in methods). Most of the 43 candidate loci were eliminated due to high levels of nonspecific amplification. One region was excluded as a paralog.

The second study is a very careful examination of anonymous primer utility across a deep turtle phylogeny (Thomson *et al*., 2008). While direct comparisons cannot be made between the two studies, a close look at Thomson *et al*. (2008) provides similar conclusions. When controlling for phylogenetic depth over 65%, 32 of 49 non-repetitive loci, had problematic amplification of 4 or more of the 9 genera of interest. Problematic amplification includes smears, multiple bands, or no amplification. Although loci have not been optimized in this

study, our results suggest that optimization will not greatly improve amplification success. In conclusion, these two studies indicate that anonymous loci may be problematic across large depths in a variety of organisms. When alternative methods to primer design are available, we advise cautious use of anonymous loci for deep phylogenetic questions.

CHAPTER 5
CONCLUSION

Thorough examination of 40 unlinked nuclear genes provides strong and independent support of ratite polyphyly and paleognath monophyly. Tinamous are consistently nested within the ratites and ostrich emerges at the basal node of the paleognath tree. This topology indicates that similarities among the ratites may be due to convergent evolution. If flight was lost multiple independent times among the ratites, convergence may account for incongruence in paleognath phylogenies. Furthermore, this topology adds flexibility for understanding ratite biogeography. Volant paleognath ancestry would no longer restrict ratites to a Gondwana origin, a hypothesis with conflicting support in the fossil record. The ratites may have had more widespread distribution, and the role of lithornithids in paleognath evolution should be re-evaluated.

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BIOGRAPHICAL SKETCH

Jordan Smith received a bachelor's degree, *cum laude,* in May of 2006. She majored in microbiology and zoology with a minor in chemistry from the University of Florida. In August of 2007, Jordan began graduate school in the Department of Zoology at the University of Florida working with Drs. Edward L. Braun and Rebecca T. Kimball. Jordan's thesis research was investigating the evolutionary relationships of paleognath birds, a controversial group important to understanding early avian evolution. In addition, Jordan was a collaborator in investigations of the population genetics of the Brown-headed Nuthatch (*Sitta pusilla*), a locally declining cooperative breeding bird